(54) Title: ENDOPLASMIC RETICULUM STRESS TRANSCRIPTION FACTOR

(57) Abstract

To provide a factor capable of efficiently regulating expression of an endoplasmic chaperone gene, a nucleic acid encoding it or a complementary strand nucleic acid thereof, a method for regulating expression of an endoplasmic reticulum chaperone gene, and a method for expressing a foreign gene. An endoplasmic reticulum stress transcription factor capable of regulating transcription–inducing activity, wherein the transcription–inducing activity is exhibited by an element having the nucleotide sequence as shown in SEQ ID NO: 1 or an element having a nucleotide sequence resulting from substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1; a method for controlling expression of an endoplasmic reticulum chaperone, comprising expressing the factor; a method for expressing a foreign protein, comprising positively regulating expression of an endoplasmic reticulum chaperone gene by the method for controlling expression; a nucleic acid encoding activated form of ATF6, activated form of CREB–RP, suppressive form of ATF6 or suppressive form of CREB–RP, or the complementary strand thereto. It is expected to be applied to treatment or prophylaxis of cancers, arteriosclerosis, cystic fibrosis, ischemic diseases, wounds or ulcers.

DESCRIPTION

ENDOPLASMIC RETICULUM STRESS TRANSCRIPTION FACTOR

5   TECHNICAL FIELD

The present invention relates to a factor capable of

efficiently regulating expression of an endoplasmic

reticulum chaperone gene, a nucleic acid encoding it or a

complementary strand nucleic acid thereof, a method for

10   regulating expression of an endoplasmic reticulum

chaperone gene, and a method for expressing a foreign

gene.

BACKGROUND ART

15   Mammalian cells, like other eukaryotic cells and

prokaryotic cells, have developed a number of homeostatic

mechanisms to cope with to various physiological and

environmental conditions that threaten their survival.

Among them, the tightly regulated synthesis of heat shock

20   proteins (HSPs) is a well-known mechanism universally

found in all organisms. In addition, the regulated

synthesis of glucose-regulated proteins (GRPs), based on a

mechanism differing from that of HSP described above, is

specifically found in eukaryotic endoplasmic reticulum

25   [Lee, A.S., *Curr. Opin. Cell Biol.* 4, 267-273 (1992);

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Morimoto, R.I. et al., *The Biology of HEAT SHOCK PROTEINS and MOLECULAR CHAPERONES*, Cold Spring Harbor Laboratory (1994)].

In mammals, eight kinds of GRPs, namely, GRP78/Bip, GRP94/ERp99, ORP150/GRP170, ERp72, GRP58/ERp60/ERp61, calreticulin, protein disulfide isomerase (PDI) and FKBP13, have been identified. These GRPs are a series of molecular chaperones or folding enzymes characteristic of the endoplasmic reticulum, each of which expression is induced by the accumulation of proteins that have failed to be folded or have undergone incorrect folding (hereinafter referred to as unfolded proteins) in the endoplasmic reticulum (endoplasmic reticulum stress) [Kozutsumi, Y. et al., *Nature* 332, 462-464 (1988); Lee, A.S., *Trends Biochem. Sci.* 12, 20-23 (1987)], and play a very important role in the folding of nascent secretory proteins and membrane proteins in the endoplasmic reticulum. Therefore, these GRPs are hereinafter generically referred to as "endoplasmic reticulum chaperones".

Expression of endoplasmic reticulum chaperones is also induced by a reagent, such as tunicamycin, which inhibits N-glycosylation of proteins, calcium ionophore A23187, which depletes calcium storage, or thapsigargin, which inhibits calcium-ATPase. These reagents are

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generally assumed to cause malfunctioning of the
endoplasmic reticulum and elicit endoplasmic reticulum
stress.

Induction of the above endoplasmic reticulum
5   chaperone by endoplasmic reticulum stress is primarily
regulated at the transcription level. Since the
endoplasmic reticulum chaperone is not induced by heat
shock stress and the promoter sequence of the endoplasmic
reticulum chaperone gene contains no heat shock elements,
10   the induction of endoplasmic reticulum chaperone is
suggested based on a regulatory mechanism differing from
that for the HSP induction. There has yet remain unknown,
however, whether an endoplasmic reticulum stress-response
is controlled by a common mechanism or by a variety of
15   mechanisms corresponding to individual endoplasmic
reticulum chaperones.

The rat GRP78 gene has already been analyzed to some
extent, and it is shown that the upstream CORE region and
the C1 region comprising the CCAAT sequence are important
20   for transcriptional regulation [Resendez, E. et al., *Mol.*
Cell. Biol. **8**, 4579-4584 (1988); Wooden, S.K. et al., *Mol.*
Cell. Biol. **11**, 5612-5623 (1991); Li, W.W. et al., *Mol.*
Cell. Biol. **14**, 5533-5546 (1994)] (see Figure 1); however,
its transcriptionally regulatory sequence yet remains
25   undetermined. In the case of yeasts, the

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transcriptionally regulatory sequence (UPRE sequence;

CAGNGTG) of the GRP78 gene of the budding yeast is

clarified [Mori, K. et al., *Genes Cells* 1, 803-817

(1996)]. Although a sequence similar to the UPRE sequence

is present upstream of the human GRP78 gene, no activity

for directing induced transcription by endoplasmic

reticulum stress has been detected in any DNA having a

sequence similar to that of the UPRE. As mentioned above,

the transcriptionally regulatory region involved in an

endoplasmic reticulum stress-response of mammals,

especially humans, still remains undetermined.

In cancer cells, endoplasmic reticulum chaperones are

expressed at high levels. For example, there has been

reported that good correlation exists between

intracellular GRP78 level and a tumor size [Cai, J.W. et

al., *J. Cell. Physiol.* 154, 229-237 (1993)], and that when

GRP78 expression is suppressed by the antisense method,

sensitivity to cytotoxic T-cell (CTL) and tumor necrosis

factor (TNF) increases [Sugawara, S. et al., *Cancer Res.*

53, 6001-6005 (1993)] and take is poor in the mouse, and

even if taking, it soon results in regression [Jamora, C.

et al., *Proc. Natl. Acad. Sci. USA* 93, 7690-7694 (1996)].

Also, it has been shown that ORP150 is strongly

induced in macrophages which infiltrate into

arteriosclerotic lesions, and that macrophages treated

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with an antisense oligonucleotide to suppress ORP150 expression show decreased viability when exposed to hypoxic conditions, especially in the presence of a denatured LDL (low-density lipoprotein) [Tsukamoto, Y. et al., *J. Clin. Invest.* 98, 1930-1941 (1996)]. Since the macrophages in arteriosclerotic lesions release cytokines, such as tumor necrosis factor, interleukin-1 (IL-1), interleukin-6 (IL-6), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β), the macrophages are positioned at the center of intercellular response in arteriosclerotic lesions and assumed to play a major role in the progression of arteriosclerosis.

Cystic fibrosis is a hereditary disease caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the most prevalent of such a mutation being the deletion of phenylalanine at residue 508 (Δ508F) [Welsh, M.J. and Smith, A.E., *Cell* 73, 1251-1254 (1993)]. CFTRΔ508F undergoes abnormal sugar chain addition and is degraded without being transported from the endoplasmic reticulum to the Golgi. However, at low temperatures, such Δ508F mutants leak from endoplasmic reticulum, are localized in the cell membrane, thereby exhibiting activity [Denning, G.M. et al., *Nature* 358, 761-764 (1992)]. Appropriately modifying the stringency

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of quality control for nascent membrane proteins in the endoplasmic reticulum would enable the localization and functioning of CTFRΔ508F in the cell membrane.

Furthermore, there have been shown that the mRNA of GRP78, as well as of HSP70, is induced in rat cerebral ischemia [Wang, S. et al., *Neurochem. Int.* **23**, 575-582 (1993); Higashi, T. et al., *Brain Res.* **650**, 239-248 (1994)], that the mRNA of GRP78 and GRP94 is induced in the hippocampal dentate gyrus when convulsive seizures are induced with kainic acid [Lowenstein, D.H. et al., *Mol. Brain Res.* **22**, 299-308 (1994); Little, E. et al., *Neuroscience* **75**, 209-219 (1996)], and that ORP150 is induced in the ischemic mouse brain [Kuwabara, K. et al., *J. Biol. Chem.* **271**, 5025-5032 (1996)]. Therefore, the endoplasmic reticulum chaperones are assumed to protectively act on damaged neurocytes by cerebral ischemia or the like.

In addition, in wounded tissues and ulcerative tissues, endoplasmic reticulum chaperones, like HSP, are expected to play an important role in repairing the damaged tissues.

On the other hand, when a foreign gene is introduced into a desired cell, to produce a useful protein, the expression of the introduced gene must be controlled, if the desired gene product exhibits cytotoxicity or affects

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cell function. In addition, when a foreign, useful

protein is expressed in a host using a recombinant DNA,

the desired protein in many cases fails to retain the

correct conformation so that the protein cannot be

5   expressed at high levels. It is suggested that the amount

of the endoplasmic reticulum chaperones and folding

enzymes in usual hosts may be insufficient to ensure ample

protein expression and formation of correct conformation.

Therefore, there is a demand for a technique for

10   enabling efficient control of the expression of the

endoplasmic reticulum chaperones.

DISCLOSURE OF INVENTION

An object of the present invention is to provide a

15   method for regulating expression of endoplasmic reticulum

chaperone genes, which is capable of increasing or

decreasing expression of the above gene; a method for

expressing a foreign protein; an endoplasmic reticulum

stress transcription factor capable of regulating

20   expression of the above gene; and a nucleic acid encoding

it, or a complementary nucleic acid thereof.

The above object and other objects of the present

invention will be apparent from the following description.

Concretely, the present invention is concerned with

25   the following:

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[1] an endoplasmic reticulum stress transcription factor
capable of regulating transcription-inducing activity,
wherein the transcription-inducing activity is exhibited
by an element having the nucleotide sequence as shown in
SEQ ID NO: 1 or an element having a nucleotide sequence
having substitution of 1 to 3 bases with other kind of
bases in the nucleotide sequence as shown in SEQ ID NO: 1;

[2] a method for controlling expression of an endoplasmic
reticulum chaperone, comprising expressing the endoplasmic
reticulum stress transcription factor as defined above in
item [1];

[3] a method for expressing a foreign protein, comprising
positively regulating expression of an endoplasmic
reticulum chaperone gene by the method as defined in item
[2];

[4] a nucleic acid encoding an activated form of ATF6, or
a complementary strand thereto;

[5] a nucleic acid encoding an activated form of CREB-RP,
or a complementary strand thereto;

[6] a nucleic acid encoding a suppressive form of ATF6,
or a complementary strand thereto; and

[7] a nucleic acid encoding a suppressive form of
CREB-RP, or a complementary strand thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 shows a nucleotide sequence of the proximal region of human GRP78 promoter [-140 to -19]. The CCAAT-like motifs designated C1 to C5 and TATA sequence are boxed and underlined, respectively. Locations of the CORE and C1 regions, and SICR and CCAAT(N$_9$)CCACG motifs are also indicated.

Figure 2 shows a tandem repeat structure of the region [-139 to -42] in the human GRP78 promoter. Three repetitive sequences in the above region are aligned, and the nucleotides conserved among at least two repeat sequences are shaded. The ERSE consensus is shown at the top.

Figure 3 shows an ERSE-like sequence (SEQ ID NOs: 5 to 30) of known mammalian and chicken GRP promoters. The nucleotides congruent with the consensus are shaded. In the figure, CRT indicates calreticulin.

Figure 4 shows locations and directions of ERSE-like sequences in various GRP promoters as indicated by closed arrows. CCAAT and TATA sequences are indicated by open arrows and small open boxes, respectively.

Figure 5 shows ERSE-like motifs in vertebrates, plants and fungi. In the figure, CRT indicates calreticulin; PDI, protein disulfide isomerase; Dm, Drosophila melanogaster; Ce, Caenorhabditis elegans; At, Arabidopsis thaliana; So, Spinacia oreracea; Rc, Ricinus

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communis; and An, *Aspergillus nigar.*

Figure 6 shows effects of disrupting ERSEs. Panel
(A) shows effects of disrupting ERSEs on human GRP78
promoter activities; panel (B) shows effects of disrupting
ERSEs on GRP94 promoter activities; and panel (C) shows
effects of disrupting ERSEs on calreticulin promoter
activities. Each of ERSE1, ERSE2 and ERSE3 of GRP78
promoter, ERSE1 and ERSE3 of GRP94 promoter, and ERSE2 and
ERSE3 of calreticulin promoter was disrupted by mutating
the respective sequences to gatcT(N_9)aacat, Ctcga(N_9)aacac,
gagcT(N_9)aacgc, atgtt(N_9)Agctc, gatcT(N_9)aactc and
atgtt(N_9)Agatc, respectively. The intact or resultant
mutant promoters were inserted into the *KpnI-XhoI* sites of
the pGL3-Basic vector. Left panel shows a schematic view
of intact or mutant ERSE-disruption constructs. Numbers
on arrows indicate a nucleotide position from the
transcription initiation site. Closed and hatched boxes
indicate the locations of an intact ERSE motif and an ERSE
motif with remarkable homology to the consensus,
respectively. Crosses indicate disrupted ERSEs. Middle
panel shows relative firefly luciferase activities by
transection into cells. Closed bars and open bars,
respectively, indicate HeLa cells transiently transfected
with each construct treated with or without 2 µg/ml
tunicamycin (in the figure, referred to as "TM") for

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16 hours. Right panel shows fold induction of induced

activities to basal levels of a reporter activity. The

relative luciferase activity and the fold induction were

determined from four independent, transient expression

5   assays.

Figure 7 shows involvement of ERSE motifs in

transcriptional induction on GRP78, GRP94 and

calreticulin. Oligonucleotides encoding each ERSE with

indicated flanking sequences as shown in the figure were

10   inserted into the BglII site of the pGL3-Promoter vector.

The relative luciferase activity and the fold induction

were determined in the manner as described in Figure 6 by

four independent, transient expression assays.

Figure 8 shows nucleotides in ERSE1 from the human

15   GRP78 promoter required for transcriptional induction.

Each nucleotide of the segment [-65 to -43] was

substituted by transversion (in the figure, substituted

nucleotides indicated by lowercase letters). Thereafter,

resulting mutated fragment was inserted into the XhoI-

20   BglII sites of the pGL2-Promoter vector. The data are

represented as the mean of four independent experiments.

Figure 9 shows nucleotides in ERSE1 from the human

GRP78 promoter required for transcriptional induction.

Each CCACG and neighboring sequences were changed to those

25   indicated by lowercase letters. Mutated fragments of the

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segment [-65 to -38] were inserted into the pGL2-Promoter

vector in the same manner as those shown in Figure 8.

Insertion mutations each comprising additional A's between

CCAAT and CCACG were also shown in lines 38 to 40. The

5   data are represented as the mean of four independent

experiments.

Figure 10 shows effects of various ER stress-inducing

reagents on ERSE-mediated induction of human GRP78, GRP94

and calreticulin. Cells transiently transfected with the

10   indicated constructs were treated for 16 hours with

2 µg/ml TM (closed bars), 1 µM A23187 (hatched bars) or

100 nM Tg (dotted bars). Each of the constructs as shown

in the figure corresponds to the constructs in Figure 6.

The data were obtained from four independent experiments.

15   Figure 11 shows a structure of a reporter plasmid

used for one-hybrid screening in yeast.

Figure 12 shows effects of a clone 3-GAL4AD fusion

protein on the reporter gene expression. An effector

plasmid carrying a gene encoding either GAL4AD protein

20   alone or clone 3-GAL4AD fusion protein was introduced into

yeast strain KMY1015 harboring a reporter plasmid of the

lacZ gene under the control of intact ERSE

(5'-CCTTCACCAATCGGCGGCCTCCACGACGG-3') or mutant ERSE (in

the figure, mutated nucleotides are indicated in

25   lowercase).

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Figure 13 shows comparison of a basic region of human ATF6. Panel (A) shows comparison with yeast *Hac1p*; Panel (B) shows comparison with members of human ATF/CREB family; and Panel (C) shows comparison with bZIP proteins.

Figure 14 shows effects of ATF6 overexpression on GRP promoters. 100 ng of an effector plasmid carrying a full-length ATF6 cDNA or plasmid vector alone was cotransfected into HeLa cells with 1 μg of a reporter plasmid containing luciferase gene fused with intact or mutant GRP promoters. The mutant promoter constructs used correspond to those of Figure 6. Closed bars and open bars indicated cells treated with or without 2 μg/ml tunicamycin (in the figure, referred to as "TM") for 16 hours, respectively. Experiments were repeated four times.

Figure 15 shows structural homology between ATF6 and CREB-RP. A region showing remarkable homology is indicated by boxes, and the identity is indicated by %.

Figure 16 shows effects of CREB-RP overexpression on GRP promoter. An effector plasmid carrying a full-length CREB-RP gene was cotransfected into HeLa cells harboring a reporter plasmid in the same manner as in Figure 14. Experiments were repeated four times. In the figure, TM indicates tunicamycin.

Figure 17 shows Northern blot hybridization analysis

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for ATF6 mRNA. HeLa cells were treated with 2 µg/ml

tunicamycin (in the figure, referred to as "TM") for

indicated period.

Figure 18 shows immunoblotting analysis of ATF6

5   protein. *in vitro* translation was carried out using

reticulocyte lysate with a control vector (lane 1) or ATF6

cDNA (lane 2). Whole cell extracts were prepared from

HeLa cells that had been untreated (lanes 3 and 5) or

treated with 2 µg/ml TM for 4 hours (lanes 4 and 6) or

10   from HeLa cells that had been transfected with a control

vector (lane 7) or an ATF6 expression plasmid (lane 8).

Proteins were detected by using anti-B03N antisera (lanes

1 to 4, 7 and 8) or anti-peptide [anti-ATF6 (N21-34)]

antisera (lanes 5 and 6). The positions of the

15   90 kDa-band (p90ATF6) and 50 kDa-band (p50ATF6) are

indicated by open and closed arrows, respectively.

Figure 19 shows the correlation of the appearance of

p50ATF6 with the intracellular UPR activity. HeLa cells

were treated with 2 µg/ml TM, 7 µM A23187 or 300 nM

20   thapsigargin (in the figure, simply referred to as "Tg")

for the indicated period. Alternatively, HeLa cells which

were treated separately from the above treated cells were

heat-shocked at 43°C for 1 hour and then recovered at 37°C

for the indicated period. Whole cell extracts were

25   prepared and analyzed by immunoblotting using an anti-B03N

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antiserum or an antiserum specific to either GRP78 or
HSP70.

Figure 20 shows immunoblotting analysis for ATF6.
HeLa cells cultured in a 60-mm dish so as to have 60%
5   confluency were incubated in the presence of 2 μg/ml
tunicamycin (TM) for the indicated period. Cells were
washed with PBS, scraped with a rubber policeman, and
suspended in 100 μl Laemmli's SDS sample buffer. After
boiling the suspension for 5 minutes, aliquots
10   corresponding to 1 x 10^5 cells were subjected to SDS-PAGE
(10% gel) and analyzed by immunoblotting using an anti-
ATF6 antibody, or an anti-KDEL antibody recognizing GRP78.
The positions of p90ATF6 and p50ATF6 are indicated by open
and closed arrows, respectively. The asterisks denote
15   non-specific bands in which a sugar chain is not added to
p90ATF6. The positions of molecular weight markers
(manufactured by Bio-Rad, prestained SDS-PAGE
preparations) are also shown.

Figure 21 shows schematic structures of ATF6 having
20   670 amino acids. The positions of the serine cluster,
basic region, and leucine zipper [Zhu et al., *Mol. Cell.
Biol.* **17**, 4957-4966 (1997)] and the transmembrane domain
identified in the present invention are indicated. The
bold line represents the region (6th to 307th amino acids)
25   in which ATF6 is fused with *Escherichia coli* maltose-bound

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protein in order to prepare an anti-AFT6 antibody. The

hydropathy index was calculated by the method of Kyte and

Doolittle, *J. Mol. Biol.* **157**, 105-132 (1982).

Figure 22 shows the distribution of p90ATF6 and

p50ATF6 in each of the fractions of HeLa cells. HeLa

cells cultured in a 175-cm^2 flask so as to have 60 to 80%

confluency were incubated in the absence (-) or presence

(+) of 2 μg/ml tunicamycin (TM) for 4 hours. Cells were

harvested, and disrupted by means of Dounce-type

homogenizer, and thereafter the disrupted product was

centrifuged at 1,000 x g for 10 minutes to obtain nuclear

pellets (in the figure, indicated by N) and supernatant

(S) essentially in the same manner as described by Dignam

et al. (1983). The resulting supernatant (S) was further

centrifuged at 100,000 x g for 1 hour to separate soluble

cytosolic fraction (C) from insoluble membrane fraction

(M). Aliquots of the indicated fraction as well as

unfractionated HeLa cells (whole cell disruption: W)

corresponding to 0.5 x 10^5 cells from which they originated

were subjected to SDS-PAGE (10% gel) and analyzed by

immunoblotting using anti-ATF6 antibody or various

antibodies indicated. The positions of p90ATF6 and

p50ATF6 are indicated as open arrows and closed arrows,

respectively.

Figure 23 shows a degree of solubilization of

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p90ATF6. 1,000 x g supernatant (S) fraction prepared from
unstressed HeLa cells obtained in the same manner as in
Figure 22 was mixed with 0.1 times by volume of one
solution selected from the group consisting of the
5   following solutions: H$_2$O, 5M NaCl, 1 M Na$_2$CO$_3$ (pH 11.0),
10% SDS, 10% Triton X-100, or 10% sodium deoxycholate
(DOC). After incubation at room temperature for 15
minutes, the mixture was centrifuged at 100,000 x g for
1 hour to separate supernatant (S) from pellets (P).
10   Thereafter, the resulting sample was subjected to SDS-PAGE
(10% gel) and immunoblotted using an anti-ATF6 antibody or
an anti-N-terminus of calnexin antibody.

       Figure 24 shows topology of p90ATF6. The 1,000 x g
supernatant (S) fraction prepared from unstressed HeLa
15   cells (50 µg proteins) was incubated with increasing
amounts of trypsin (0 µg for lanes 1, 5 and 9, 0.1 µg for
lanes 2, 6 and 10, 0.3 µg for lanes 3, 7 and 11, and
1.0 µg for lanes 4, 8 and 12) at room temperature for
15 minutes. Digestion was terminated by addition of an
20   equal volume of 2 x Laemmli's SDS sample buffer followed
by boiling for 5 minutes. Samples were subjected to SDS-
PAGE (10% gel), and analyzed by immunoblotting with anti-
ATF6 antibody (lanes 1 to 4), anti-N-terminus of calnexin
antibody (Calnexin-N, lanes 5 to 8), or anti-C-terminus of
25   calnexin (Calnexin-C, lanes 9 to 12) antibody. The

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positions of p90ATF6 are indicated by an arrow. The

positions of a full-length calnexin and its truncated form

lacking the cytoplasmic domain are shown schematically.

5

Figure 25 shows schematic structures of full-length

ATF6 cDNA, ATF6(670), which is inserted into mammalian

expression vector pCGN and various deletion mutants. In

the figure, the positions of the basic region, the leucine

zipper region and the transmembrane domain are indicated.

HA epitope is bound to N-terminal each of various deletion

10   mutants.

Figure 26 shows comparison of mobility on SDS-PAGE

for each of various C-terminal deletion mutants with that

of p90ATF6 or p50ATF6. HeLa cells in a 60-mm dish were

transiently transfected with pCGN vector alone (Vec),

15   expression plasmids for C-terminal deletion mutants

pCGN-ATF6(670), pCGN-ATF6(402), pCGN-ATF6(373),

pCGN-ATF6(366), or pCGN-ATF6(330). Whole proteins were

directly extracted with 1 × Laemmli's SDS sample buffer,

and the extract was subjected to SDS-PAGE (10% gel) and

20   analyzed by immunoblotting with an anti-ATF6 antibody.

The positions of p90ATF6 and p50ATF6 are indicated by open

and closed arrows, respectively.

Figure 27 shows the analytic results of

transcription-activating domain of ATF6. Left panel shows

25   schematic structures of ATF6 and fusion proteins between

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various ATF6 subregions and the DNA-binding domain (1st to 147th amino acids) of yeast *Gal4p* (GAL4DB). In the figure, the dotted lines denote the region deleted from the construct. The positions of the basic leucine zipper region (bZIP) and the transmembrane domain (TMD) are indicated. Right panel shows transcription activities of various fusion proteins. HeLa cells in a 24-well plate were transiently transfected with each of the fusion protein expression plasmids together with the reporter plasmid pG5luc containing five *Gal4p* binding sites upstream of the firefly luciferase gene. Constitutively expressed luciferase activities were determined and normalized as described in Examples. The relative activities are indicated as the mean ± standard deviations (bars) from four independent experiments (triplicate determinations). The positive control supplied by the manufacturer (pBIND-Id plus pACT-MyoD control vectors; manufactured by Promega) showed the relative activity of 4.6 ± 0.3 in this assay.

Figure 28 shows effects of overexpression on full-length ATF6, C-terminal deletion mutants and transcription-activating domain deletion mutant on ERSE-mediated transcription. HeLa cells in a 24-well plate were transiently transfected with a reporter plasmid pGL-G78(-132) capable of expressing firefly luciferase

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gene under the control of human GRP78 promoter, together

with pCGN vector alone (Vec) or each of ATF6 expression

plasmids. Transfected cells were treated with (closed

bars) or without (open bars) 2 μg/ml tunicamycin (TM) for

5   16 hours prior to measuring luciferase activity. The

relative activities are indicated as the mean ± standard

deviations (bars) from four independent experiments

(triplicate determinations).

       Figure 29 shows the results of immunoblot analysis

10   for CREB-RP. In the same manner as the method described

in Figure 20, the analysis was carried out by applying a

cell extract to SDS-PAGE, and then immunoblotting the

resulting gel using an anti-CREB-RP antibody or an

anti-ATF6 antibody or an anti-KDEL antibody.

15   Figure 30 shows effects of overexpression of

p60CREB-RP and p50ATF6 on ERSE-mediated transcription. In

a 96-well plate, the HeLa cells were transiently

transformed with a reporter plasmid pGL-G78(-132) for

expressing firefly luciferase gene under the control of a

20   human GRP78 promoter together with pcDNA3.1 vector which

has no inserts or with ATF6(1-373) expression plasmid or

with CREB-RP(1-389) expression plasmid. Relative activity

is indicated by the mean ± standard deviation (bars),

based on four independent experiments (each triplicate

25   determinations).

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Figure 31 shows effects of overexpression of a
deletion mutant of CREB-RP transcription-activating domain
on ERSE-mediated transcription. In a 96-well plate, the
HeLa cells were transiently transformed using a reporter
plasmid pGL-G78(-132) for expressing firefly luciferase
gene under the control of a human GRP78 promoter together
with pcDNA3.1 vector which has no inserts or with
CREB-RP(308-386) expression plasmid. Transfected cells
were treated with (closed bars) or without 2 µg/ml
tunicamycin (open bars) for 16 hours before luciferase
activities were determined. Relative activity is
indicated by the mean ± standard deviation (bars), based
on four independent experiments (each triplicate
determinations).

Figure 32 shows effects of overexpression of deletion
mutants of CREB-RP transcription-acting domain,
CREB-RP(151-389) and CREP-RP(81-389) on ERSE-mediated
transcription. In the figure, closed bars and open bars
indicate the same as those in Figure 31.

BEST MODE FOR CARRYING OUT THE INVENTION

In the present specification, the term "endoplasmic
reticulum chaperone" refers to a series of proteins
characteristic of endoplasmic reticulum, wherein
expression of the above proteins is induced by endoplasmic

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reticulum stress, and the above proteins are a protein or

enzyme having an activity for promoting or catalyzing

folding or formation of conformation of secretory proteins

and membrane proteins. The endoplasmic reticulum

5   chaperone includes, for example, GRP78, GRP94, ORP150,

ERp72, GRP58, calreticulin, PDI, FKBP13, and the like.

The endoplasmic reticulum chaperone also encompasses

proteins derived from animals such as mammals, and

proteins derived from plants.

10   In addition, a gene encoding the above endoplasmic

reticulum chaperone refers to herein as "endoplasmic

reticulum chaperone gene."

(1)   Endoplasmic Reticulum Stress Transcription Factor

15   Capable of Regulating Expression of Endoplasmic

Reticulum Chaperone Gene and Nucleic Acid Encoding It

The phrase "endoplasmic reticulum stress

transcription factor capable of regulating expression of

endoplasmic reticulum chaperone genes" refers to a factor

20   capable of regulating expression by interaction with an

endoplasmic reticulum stress-response element (hereinafter

simply referred to as "ERSE") which is present on the

above endoplasmic reticulum chaperone gene. Therefore,

the endoplasmic reticulum stress transcription factor of

25   the present invention can exhibit an excellent effect for

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regulating expression of the above endoplasmic reticulum
chaperone genes at once.

Here, the term "ERSE" refers to an element having
activity for directing induced transcription by
5   endoplasmic reticulum stress, wherein the element is an
element having a nucleotide sequence as shown in
SEQ ID NO: 1 or an element having a nucleotide sequence
having substitution of 1 to 3 bases with other kind of
bases in the nucleotide sequence as shown in SEQ ID NO: 1.
10   Concretely, the above endoplasmic reticulum stress
transcription factor includes an endoplasmic reticulum
stress transcription factor capable of regulating
transcription-inducing activity, wherein the
transcription-inducing activity is exhibited by an element
15   having the nucleotide sequence as shown in SEQ ID NO: 1,
or an element having a nucleotide sequence having
substitution of 1 to 3 bases with other kind of bases in
the nucleotide sequence as shown in SEQ ID NO: 1.

The phrase "nucleotide sequence having substitution
20   of 1 to 3 bases with other kind of bases" means a
naturally occurring nucleotide sequence having
substitution of 1 to 3 bases with other kind of bases and
a nucleotide sequence resulting from artificial
substitution of 1 to 3 bases with other kind of bases.

25   The endoplasmic reticulum stress transcription factor

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includes, for example, bZIP transcription factor,
concretely ATF6 [Hai, T.W. et al., *Genes Dev.* 3, 2083-2090
(1989)], CREB-RP [Min, J. et al., *Genomics* 30, 149-156
(1995)], XBP-1/TREB5 [Liou, H.C. et al., *Science* 247,
1581-1584 (1990), Yoshimura, T. et al., *EMBO J.* 9,
2537-2542 (1990)], and the like.

The nucleotide sequence and the amino acid sequence
of the above ATF6 are shown in SEQ ID NOs: 31 and 32,
respectively. In addition, the nucleotide sequence and
the amino acid sequence of the above CREB-RP are shown in
SEQ ID NOs: 33 and 34, respectively.

The endoplasmic reticulum stress transcription factor
encompasses a factor capable of regulating transcription-
inducing activity which is exhibited by the above
endoplasmic reticulum stress-response element, wherein the
factor comprises a polypeptide which can be encoded by a
nucleic acid selected from the group consisting of:
(A) a nucleic acid having the nucleotide sequence as
shown in SEQ ID NO: 31;
(B) a nucleic acid having the nucleotide sequence as
shown in SEQ ID NO: 33;
(C) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of one or
more bases in the nucleotide sequence of the nucleic acid
(A) or (B); and

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(D) a nucleic acid capable of hybridizing under stringent conditions to the strand complementary to any one of nucleic acids (A) to (C).

In the present specification, the phrase "having substitution, deletion, addition or insertion of one or more bases" refers to a state in which one or more bases are naturally or artificially substituted, deleted, added or inserted. In addition, "one or more bases" can be selected in a range capable of regulating transcription-inducing activity exhibited by an endoplasmic reticulum stress-response element.

Further, the term "nucleic acid" refers, for example, to a DNA, and an RNA corresponding thereto.

The term "stringent conditions" includes, for example, conditions described in *Molecular Cloning: A Laboratory Manual, Second Edition* [Sambrook, J. et al. (1989)], and the like.

The endoplasmic reticulum stress transcription factor further encompasses, as long as the factor is capable of regulating transcription-inducing activity exhibited by an endoplasmic reticulum stress-response element, a polypeptide comprising an amino acid sequence having substitution, deletion, addition, or insertion of one or more amino acids in the amino acid sequence as shown in each of SEQ ID NOs: 32 and 34.

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In the present specification, "having substitution, deletion, addition, or insertion of one or more amino acids" refers to a state in which one or more amino acids are naturally or artificially substituted, deleted, added

5   or inserted. In addition, "one or more amino acids" can be selected in a range capable of regulating transcription-inducing activity exhibited by an endoplasmic reticulum stress-response element.

In addition, the present invention encompasses an

10   endoplasmic reticulum stress transcription factor which can be obtained as follows.

A method for obtaining an endoplasmic reticulum stress transcription factor capable of enhancing expression of endoplasmic reticulum chaperone genes

15   (hereinafter referred to as "activated form of an endoplasmic reticulum stress transcription factor") includes, but not particularly limited to, a method comprising comparing an expression level of a reporter gene which is incorporated downstream of ERSE described as

20   follows in the absence or presence of endoplasmic reticulum stress by a one-hybrid method using ERSE and a yeast host, whereby obtaining the factor using an increase of the expression level as an index.

On the other hand, an endoplasmic reticulum stress

25   transcription factor for decreasing expression of

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endoplasmic reticulum chaperone genes (hereinafter

referred to as "suppressive form of an endoplasmic

reticulum stress transcription factor") can be obtained by

comparing an expression level of a reporter gene which is

5   incorporated downstream of ERSE in the absence or presence

of endoplasmic reticulum stress, whereby obtaining the

factor using a decrease in the expression level as an

index in the same manner as the above.

      The activated form of an endoplasmic reticulum stress

10   transcription factor includes, for example, ATF6, and the

like.

      It is suggested in the following examples of the

present specification that the above ATF6 is a membrane

protein of the endoplasmic reticulum, and the ATF6 is a

15   bZIP transcription factor having a characteristic of

increasing expression of an endoplasmic reticulum

chaperone. Transcription-increasing activity of

endoplasmic reticulum chaperone genes in which ERSE having

intact CCAAT and CCACG region of the nucleotide sequence

20   as shown in SEQ ID NO: 1 is present can be demonstrated by

the presence of the above ATF6.

      Although the ATF6 is usually a protein having a

molecular weight of about 90 kDa (hereinafter referred to

as "p90ATF6"), the p90ATF6 is converted into a 50 kDa

25   activated form of protein (hereinafter referred to as

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p50ATF6) in the presence of endoplasmic reticulum stress.
It is strongly suggested in the following examples of the
present specification that the above p50ATF6 is a portion
for N-terminal region of p90ATF6, and is localized in the

5   nucleus. p50ATF6 is preferable because the p50ATF6
enhances an activity for increasing expression of
endoplasmic reticulum chaperone genes.

    The term "endoplasmic reticulum stress" refers to
phenomena of accumulation of unfolded proteins in the

10   endoplasmic reticulum under various conditions preventing
correct folding of proteins. Concretely, the endoplasmic
reticulum stress can be caused by a treatment with, for
instance, glucose depletion, tunicamycin [Kozutsumi, Y. et
al., Nature 332, 462-464 (1988)], calcium ionophore A23187

15   [Watowich, S.K., Mol. Cell. Biol. 11, 5612-5623 (191)],
thapsigargin [Li, W.W., J. Biol. Chem. 268, 12003-12009
(1993)], 2-deoxyglucose, hypoxia and the like.

    Conditions for obtaining p50ATF6 by the above
endoplasmic reticulum stress are not particularly limited.

20   For example, when tunicamycin is used, p90ATF6 can be
converted into p50ATF6 by treating cells with 0.5 to
8 μg/ml tunicamycin for 2 to 8 hours to induce endoplasmic
reticulum stress.

    In addition, the activated form of ATF6 includes a

25   polypeptide comprising an N-terminal region (an entire or
partial portion of a region of 1st to 373rd amino acids or a

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region of 1st to 366th amino acids). From the viewpoint of

sufficiently exhibiting activity for increasing expression

of endoplasmic reticulum chaperone genes, the activated form

of ATF6 includes a polypeptide comprising preferably a

5   region of 1st to 43rd amino acids, more preferably a region

of 1st to 150th amino acids. Such an activated form of ATF6

can be prepared, for instance, by introducing stop codon at

the position of termination for a desired amino acid region.

Alternatively, examples of the suppressive form of an

10   endoplasmic reticulum stress transcription factor include

suppressive form of ATF6, CREB-RP, and the like. Those

factors have a property for decreasing expression of

endoplasmic reticulum chaperone genes.

The above suppressive form of ATF6 is a polypeptide

15   resulting from disruption of an entire or partial portion of

a region of 1st to 150th amino acids from ATF6 or activated

form of ATF6. Such a suppressive form of ATF6 has

properties of acting as dominant negative, and decreasing

expression of endoplasmic reticulum chaperone genes.

20   Here, the term "disruption of an entire or partial

portion" refers to a state in which the function possessed

by the activated form of the transcription factor cannot be

exhibited by mutations such as deletion, insertion,

substitution, and the like.

25   Although the above CREB-RP is usually a 110 kDa

protein (hereinafter referred to as "p110CREB-RP"), the

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p110CREB-RP is converted into a 60 kDa protein
(hereinafter referred to as "p60CREB-RP") in the presence
of endoplasmic reticulum stress, in the same manner as
conversion of p90ATF6 into p50ATF6 by endoplasmic
reticulum stress. Surprisingly, differing from
p110CREB-RP, p60CREB-RP has an activity for increasing
expression of endoplasmic reticulum chaperone genes and
acts as an activated form of an endoplasmic reticulum
stress transcription factor. That is, in addition to the
above ATF6 (p90ATF6 and p50ATF6), the activated form of an
endoplasmic reticulum stress transcription factor includes
p60CREB-RP.

Furthermore, a polypeptide resulting from deletion of
an entire or partial portion of a region of 1st to 307th
amino acids from the above activated form of CREB-RP has
properties of acting as dominant negative, and is included
in the suppressive form of an endoplasmic reticulum stress
transcription factor.

The presence or absence of expression of endoplasmic
reticulum chaperone genes by the above endoplasmic
reticulum stress transcription factor (for example, bZIP
transcription factor, and the like) can be confirmed, for
instance, by quantifying mRNA to evaluate the presence or
absence of transcription. In other words, whether or not
the desired gene is transcribed can be determined by
extracting an RNA from cells, and subjecting the resulting

RNA to Northern blot hybridization or RNA protection
assay. The transcription level of the corresponding mRNA
also can be relatively evaluated by introducing a plasmid
resulting from incorporation of a fusion gene of the above

5   endoplasmic reticulum chaperone gene with
a reporter gene such as chloramphenicol acetyltransferase
(hereinafter referred to as "CAT") gene or luciferase
gene; and then measuring an activity for the reporter gene
product CAT or luciferase.

10   The above endoplasmic reticulum stress transcription
factor (for example, bZIP transcription factor, and the
like) can be expressed, according to conventional methods,
by using cells resulting from incorporation of a DNA
comprising a gene encoding the above endoplasmic reticulum

15 stress transcription factor into a chromosome of a host
cell; or cells resulting from incorporation of the same
DNA into a vector commonly used, and thereafter
introducing the resulting vector into a host cell.

There may be used a gene encoding endoplasmic

20 reticulum stress transcription factor in which suitable
promoter, and the like are placed. The above promoter
includes, for example, SV40 promoter, cytomegalovirus
promoter, retrovirus LTR promoter, β-actin promoter, yeast
ADH1 promoter, yeast GAP-DH promoter, and the like.

25   When the above gene is incorporated into a vector,

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the above vector commonly used includes plasmids, cosmids,

viruses, and the like. Concretely, the vector includes,

but not limited to, pKCR, pcDL-SRα, pCAGGS, retrovirus

vector, adenovirus vector, adeno-associated virus vector,

5   vectors for yeast: YIp, YCp, YEp, YRp derivative plasmids,

and the like.

The above host, but not limited to, includes for

example, HeLa cells, CHO cells, FM3A cells, L cells,

BALB/c3T3 cells, BHK cells, ES cells, yeast *Saccharomyces*

10   *cervisae*, and the like.

A method for introducing a DNA carrying a gene

encoding the above endoplasmic reticulum stress

transcription factor into a host cell includes, but not

limited to, conventional methods such as calcium phosphate

15   method, electroporation method, lipofection method, and

DEAE dextran method.

In the endoplasmic reticulum transcription factor of

the present invention, there may be a case where the

extent of exhibiting the effect of increase or decrease in

20   expression is different depending upon the ERSE nucleotide

sequence in the gene.

Examples of the ERSE include an element

[CCAAT(N)₉CCACG] having the nucleotide sequence as shown in

SEQ ID NO: 1. By analyzing the transcriptional regulatory

25   region (SEQ ID NO: 4, Figure 1) of a gene encoding a kind

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of GRP, human GRP78 protein, the above sequence was clarified for the first time that the sequence is a region involved in the transcriptional regulation by endoplasmic reticulum stress and obtained thereby.

5   The nucleotide sequence as shown in SEQ ID NO: 1 is a well preserved sequence in ERSE1 (SEQ ID NO: 5) of human GRP78, ERSE1 (SEQ ID NO: 6) of murine GRP78, ERSE1 (SEQ ID NO: 7) of rat GRP78, ERSE1 (SEQ ID NO: 8) of human GRP94, ERSE1 (SEQ ID NO: 9) of chicken GRP94, ERSE3

10   (SEQ ID NO: 10) of human GRP94, ERSE3 (SEQ ID NO: 11) of chicken GRP94, ERSE3 (SEQ ID NO: 12) of human calreticulin, ERSE3 (SEQ ID NO: 13) of murine calreticulin, and the like.

In addition, the ERSE is a nucleotide sequence having

15   substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1, which may be an element capable of exhibiting transcription-inducing activity by endoplasmic reticulum stress. The above element may be a DNA resulting from substitution of

20   1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1 by genetic engineering techniques, or it may be a naturally occurring DNA having a nucleotide sequence having substitution of 1 to 3 bases. Examples of the naturally occurring DNAs include ERSE2

25   (SEQ ID NO: 14) of human GRP78, ERSE2 (SEQ ID NO: 15) of

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murine GRP78, ERSE2 (SEQ ID NO: 16) of rat GRP78, ERSE3

(SEQ ID NO: 17) of human GRP78, ERSE3 (SEQ ID NO: 18) of

murine GRP78, ERSE3 (SEQ ID NO: 19) of rat GRP78, ERSE2

(SEQ ID NO: 20) of human GRP94, ERSE4 (SEQ ID NO: 21) of

5   human GRP94, ERSE2 (SEQ ID NO: 22) of chicken GRP94, ERSE1

(SEQ ID NO: 23) of human calreticulin, ERSE2

(SEQ ID NO: 24) of human calreticulin, ERSE2

(SEQ ID NO: 25) of murine calreticulin, ERSE1

(SEQ ID NO: 26) of murine ERp72, ERSE2 (SEQ ID NO: 27) of

10   murine ERp72, ERSE1 (SEQ ID NO: 28) of human protein

disulfide isomerase, ERSE2 (SEQ ID NO: 29) of human

protein disulfide isomerase, ERSE1 (SEQ ID NO: 30) of

human GRP58, and the like. In the ERSE, similar sequences

are found in vertebrates, plants, fungi, and the like

15   (Figure 5).

       In the ERSE, the expression can be further

effectively controlled by having coexistence of three

elements ERSEs 1 to 3 as shown in SEQ ID NOs: 1 to 3.

       A method for obtaining the ERSE is not particularly

20   limited thereto, and the ERSE can be obtained as described

in Examples set forth below.

       The nucleic acid for encoding the endoplasmic

reticulum stress transcription factor has an excellent

characteristic that the nucleic acid can be used in gene

25   therapies for diseases such as ischemic diseases and

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cancers. For instance, there are expected that the ischemic diseases can be treated by positively controlling the expression of the endoplasmic reticulum chaperone gene (increase in expression level), and conversely the cancers

5   can be treated by negatively controlling the expression (decrease in expression level).

When expression of the endoplasmic reticulum chaperone gene is positively controlled, as the nucleic acid encoding the endoplasmic reticulum stress

10   transcription factor, there can be used a nucleic acid encoding an activated form of ATF6 or a nucleic acid encoding an activated form of CREB-RP.

Concrete examples of the nucleic acid encoding an activated form of ATF6 include any one of nucleic acids

15   selected from the group consisting of:

(a)   a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 373 in SEQ ID NO: 32;

(b)   a nucleic acid having a nucleotide sequence encoding

20   an amino acid sequence as shown in amino acid numbers: 1 to 366 in SEQ ID NO: 32;

(c)   a nucleic acid having a nucleotide sequence as shown in base numbers: 69 to 1187 in SEQ ID NO: 31;

(d)   a nucleic acid having a nucleotide sequence as shown

25   in base numbers: 69 to 1166 in SEQ ID NO: 31;

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(e)　a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (a) to (d); and

(f)　a nucleic acid capable of hybridizing to a strand complementary to the nucleic acid of any one of (a) to (e) under stringent conditions,

or the complementary strand thereto.　In addition, concrete examples of the nucleic acid encoding an activated form of CREB-RP include any one of nucleic acids selected from the group consisting of:

(g)　a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 389 in SEQ ID NO: 34;

(h)　a nucleic acid having a nucleotide sequence as shown in base numbers: 47 to 1213 in SEQ ID NO: 33;

(i)　a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (g) and (h); and

(j)　a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (g) to (i) under stringent conditions,

or the complementary strand thereto.

When expression of the endoplasmic reticulum chaperone gene is negatively regulated (decrease in

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expression level), as the nucleic acid encoding the

endoplasmic reticulum stress transcription factor, there

can be used a strand nucleic acid complementary to the

nucleic acid encoding an activated form of ATF6, a nucleic

5   acid strand complementary to the nucleic acid encoding an

activated form of CREB-RP, a nucleic acid encoding a

suppressive form of ATF6, a nucleic acid encoding a

suppressive form of CREB-RP, and the like.

        Concretely, as the complementary strand to the

10   nucleic acid encoding an activated form of ATF6, there can

be cited complementary strand to the nucleic acids

selected from the group consisting of (a) to (f) above.

The complementary strand to the nucleic acid encoding an

activated form of CREB-RP includes complementary strand to

15   the nucleic acids selected from the group consisting of

(g) to (j) above. As the nucleic acid encoding a

suppressive form of ATF6, there can be cited nucleic acids

selected from the group consisting of:

        (k) a nucleic acid having a nucleotide sequence encoding

20   an amino acid sequence as shown in amino acid numbers: 151

to 670 in SEQ ID NO: 32;

        (l) a nucleic acid having a nucleotide sequence encoding

an amino acid sequence as shown in amino acid numbers: 151

to 373 in SEQ ID NO: 32;

25   (m) a nucleic acid having a nucleotide sequence encoding

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an amino acid sequence as shown in amino acid numbers: 151 to 366 in SEQ ID NO: 32;

(n) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 2078 in SEQ ID NO: 31;

5   (o) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 1187 in SEQ ID NO: 31;

(p) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 1166 in SEQ ID NO: 31;

(q) a nucleic acid having a nucleotide sequence having

10   substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (k) to (p); and

(r) a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (k) to (q) under stringent conditions,

15   or the complementary strand thereto. The nucleic acid encoding a suppressive form of CREB-RP includes nucleic acids selected from the group consisting of:

(s) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 308

20   to 386 in SEQ ID NO: 34;

(t) a nucleic acid having a nucleotide sequence as shown in base numbers: 968 to 1204 in SEQ ID NO: 33;

(u) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 151

25   to 389 in SEQ ID NO: 34;

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(v) a nucleic acid having a nucleotide sequence as shown
in base numbers: 497 to 1213 in SEQ ID NO: 33;

(w) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 81

to 389 in SEQ ID NO: 34;

(x) a nucleic acid having a nucleotide sequence as shown
in base numbers: 287 to 1213 in SEQ ID NO: 33;

(y) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of at least

one base in the nucleic acid of any one of (s) to (x); and

(z) a nucleic acid capable of hybridizing to the strand
complementary to the nucleic acid of any one of (s) to (y)
under stringent conditions,

or the complementary strand thereto.

The substances having activity for suppressing
expression of the endoplasmic reticulum chaperones [for
instance, a suppressive form of ATF6, nucleic acids of (k)
to (r), complementary strands to nucleic acids of (a) to
(f), a suppressive form of CREB-RP, nucleic acids of (s)

to (z), complementary strands to nucleic acids of (g) to
(j), and the like] are expected to be used as a
therapeutic drug or a prophylactic drug for cancers,
arteriosclerosis or cystic fibrosis. In addition, the
substances having activity for inducing expression of the

endoplasmic reticulum chaperones [for instance, an

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activated form of ATF6, nucleic acids of (a) to (f), an

activated form of CREB-RP, nucleic acids of (g) to (j),

and the like] are expected to be used as a therapeutic

drug or a prophylactic drug for ischemic diseases, wounds

5   or ulcers.

When the substance having activity for suppressing

expression of the endoplasmic reticulum chaperones or the

substance having activity for inducing expression of the

endoplasmic reticulum chaperones is used as a therapeutic

10   drug or prophylactic drug, its administration form

includes oral administration, inhalant administration,

intravenous injection, subcutaneous injection, and the

like.

From the viewpoint of easiness in the introduction of

15   the nucleic acid or the complementary strand thereto into

cells, the method for administering the nucleic acid or

the complementary strand thereto of the present invention

includes a method of administration of a construct

resulting from incorporation of the nucleic acid or the

20   complementary strand thereto into a virus vector by means

such as oral administration, inhalant administration,

intravenous injection, subcutaneous injection, and the

like; a method for directly intramuscularly administering

a composition comprising an expression plasmid harboring

25   the nucleic acid or the complementary strand thereto (DNA

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vaccine method); liposome method; lipofection method;

microinjection method; calcium phosphate method;

electroporation method, and the like.

   The nucleic acid or the complementary strand thereto

5 of the present invention may be chemically modified

nucleic acids, including, for instance, phosphothioates,

phosphodithioates, trialkylphosphoric acid esters, alkyl

phosphonates, alkylphosphoamidates, and the like.

10 (2)   Method for Controlling Expression of Endoplasmic

      Reticulum Chaperone

      One of the great features of a method for controlling

expression of an endoplasmic reticulum chaperone resides

in that the endoplasmic reticulum stress transcription

15 factor, for instance, bZIP transcription factor, or the

like) is expressed. According to the method for

controlling expression of endoplasmic reticulum chaperones

of the present invention, there is exhibited an excellent

effect in that treatment or prophylaxis of cancers,

20 arteriosclerosis, cystic fibrosis, ischemic diseases,

wounds or ulcers is made possible by controlling

expression of endoplasmic reticulum chaperone genes using

the endoplasmic reticulum stress transcription factor. In

addition, according to the method for controlling

25 expression of endoplasmic reticulum chaperone genes of the

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present invention, there is exhibited an excellent effect

in that a correct conformation can be maintained and a

desired protein can be exhibited at a high level in the

expression of a foreign protein by a recombinant DNA.

5   Since the endoplasmic reticulum stress transcription

factor of the present invention can control expression of

all of the endoplasmic reticulum chaperone genes at once,

the method using the endoplasmic reticulum stress

transcription factor of the present invention is expected

10   to have remarkably excellent controlling effects as

compared to the method of controlling expression of each

of the individual endoplasmic reticulum chaperone genes.

In the present invention, expression of endoplasmic

reticulum chaperone genes can be controlled by regulating

15   an expression level of the endoplasmic reticulum stress

transcription factor within the cell, or the activity for

positively or negatively regulating expression of

endoplasmic chaperone genes can be adjusted by selecting

the endoplasmic reticulum stress transcription factor to

20   be expressed in the cells.

When expression of the endoplasmic reticulum

chaperone genes is controlled by regulating the expression

level of the endoplasmic reticulum stress transcription

factor, the expression level can be regulated by

25   introducing into cells a DNA encoding the endoplasmic

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reticulum stress transcription factor, or a DNA encoding

antisense RNA of the endoplasmic reticulum stress

transcription factor, or an antisense oligonucleotide.

When expression of the endoplasmic reticulum

5   chaperone genes is regulated positively (increase in

expression level) or negatively (decrease in expression

level) in accordance with the selection of the endoplasmic

reticulum stress transcription factor to be expressed, the

expression can be regulated by, for instance, selecting an

10   endoplasmic reticulum stress transcription factor from a

bZIP transcription factor ATF6, CREB-RP, XBP-1/TREB5, or

the like in accordance with the direction (positive or

negative) of the regulation of expression. Besides the

above, there may be appropriately selected the endoplasmic

15   reticulum stress transcription factors described in item

(1) above (for instance, bZIP transcription factors), or

other transcription factors depending upon the

characteristics owned by nucleic acids.

When the expression level of the endoplasmic

20   reticulum chaperone gene is increased, for instance, one

obtained by expressing AFT6 within the cell as p90ATF6, to

form p50ATF6 by endoplasmic reticulum stress may be used,

or one obtained by expressing a DNA encoding p50ATF6 may

be used. Further, there may be used one obtained by

25   expressing a DNA encoding p60CREB-RP.

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In addition, when the ATF6 is used, a polypeptide containing an N-terminal region (an entire or partial portion of a region of 1st to 373th amino acids, or of an entire or partial portion of a region of 1st to 366th

5   amino acids) can be used. From the viewpoint of sufficiently exhibiting activity of increasing expression of the endoplasmic reticulum chaperone genes, there may be employed one obtained by expressing within the cells a DNA encoding a polypeptide preferably containing a region of

10   1st to 43rd amino acids, more preferably a region of 1st to 150th amino acids.

Further, when the activated form of CREP-RP is used, there may be employed a polypeptide containing an N-terminal region (an entire or partial portion of a

15   region of 1st to 389th amino acids), or one obtained by expressing a DNA encoding the above polypeptide within the cell.

On the other hand, when the expression level of the endoplasmic reticulum chaperone genes is decreased, a

20   polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 150th amino acids from ATF6 or the activated form of ATF6 is used as a dominant negative form, and whereby the expression level of the endoplasmic reticulum chaperone genes can be

25   decreased. In this case, there may be used one obtained

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by expressing a DNA encoding the above polypeptide within the cell.

In addition, when the expression level of the endoplasmic reticulum chaperone genes is decreased, a
5   polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 307th amino acids from the activated form of CREB-RP is used as a dominant negative form, and whereby the expression level of the endoplasmic reticulum chaperone genes can be decreased.
10   In this case, there may be used one obtained by expressing a DNA encoding the above polypeptide within the cell.

(3)   Method for Expressing Foreign Protein

There is further provided a method for expressing a
15   foreign protein comprising the method of the present invention for controlling expression of endoplasmic reticulum chaperon genes. The method for expressing a foreign protein is also encompassed in the present invention.
20   When utilized for control of expression of a foreign gene, 1) a gene encoding the activated form of endoplasmic reticulum stress transcription factor may be simultaneously expressed with a foreign gene located downstream of a promoter for the endoplasmic reticulum
25   chaperone gene, or 2) a gene encoding the activated form

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of endoplasmic reticulum stress transcription factor may

be simultaneously expressed with a DNA in which an

appropriate promoter, a foreign gene, or the like is

located downstream of the DNA containing an ERSE portion

5   of the endoplasmic reticulum chaperone gene.

1)   Embodiment where a gene encoding the activated form

of endoplasmic reticulum stress transcription factor is

simultaneously expressed with a foreign gene located

10   downstream of a promoter for the endoplasmic reticulum

chaperone gene

In this embodiment, a vector carrying a gene encoding

the activated form of endoplasmic reticulum stress

transcription factor may be coexpressed with a vector

15   carrying a foreign gene located downstream of the

endoplasmic reticulum chaperone gene. Alternatively, a

foreign gene may be expressed by introducing the vector

carrying the foreign gene into a previously prepared host

capable of controlling expression of a gene encoding the

20   activated form of an endoplasmic reticulum stress

transcription factor by endoplasmic reticulum stress.

The vector includes, but not particularly limited to,

the vectors mentioned above.

The host used for expression includes, but not

25   particularly limited to, the hosts mentioned above.

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The method of transfection using a vector includes, but not particularly limited to, the conventional methods mentioned above.

5   2)   Embodiment where a gene encoding the activated form of an endoplasmic reticulum stress transcription factor is simultaneously expressed with a DNA in which an appropriate promoter, a foreign gene, or the like is located downstream of the DNA containing an ERSE portion

10   of the endoplasmic reticulum chaperone gene

The promoter includes not only promoters derived from human GRP78 gene, human GRP94 gene, and human calreticulin gene, but also any foreign promoters. Concrete examples of the foreign promoters include SV40 promoter,

15   cytomegalovirus promoter, retrovirus LTR promoter, β-actin promoter, and the like.

The DNA comprising the ERSE portion of the endoplasmic reticulum chaperone gene can be located at any positions upstream of the transcriptional initiation

20   point, and it is preferably located within 600 bases from the transcriptional initiation point. Further, the orientation of the DNA may be + or -.

The DNA comprising the ERSE portion of the endoplasmic reticulum chaperone gene can be used for an

25   expression vector by incorporating the DNA into generally

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employed plasmids, cosmids, viruses, and the like.

The cells used for expression include the cells

mentioned above. In addition, the transfection of the

expression vector may be carried out by a conventional

5   method of transfection mentioned above.

EXAMPLES

The present invention will be described in further

detail by means of the following Examples, without

10   intending to limit the scope or spirit of the present

invention thereto.

Example 1: Isolation of ERSE

(1)   Cell Culture

15   HeLa cells (ATCC CCL2) were cultured in Dulbecco's

modified Eagle's medium (glucose at 4.5 g/l) supplemented

with 10% fetal calf serum, 2 mM glutamine, 100 unit/ml

penicillin and 100 µg/ml streptomycin, at 37°C in a

humidified 5% CO_2/95% air atmosphere.

20

(2)   Construction of Reporter Plasmids for Isolating ERSE

Recombinant DNA techniques were performed according

to the standard procedures described in *Molecular Cloning:*

A Laboratory Manual Second Edition, Cold Spring Harbour

25   Laboratory, Published in 1989, and the like.

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A 311 bp fragment of a human GRP78 promoter ([-304 to +7] region; the nucleotide position of the transcription initiation site is defined as +1) was amplified by PCR method with genomic DNA of HeLa cells as a template using two oligonucleotides as primers for + strand and - strand, wherein the oligonucleotides were prepared on the basis of the sequence of the human GRP78 gene disclosed by Ting et al. (mentioned above). The resulting amplified fragment was inserted into the KpnI-XhoI sites of the pGL3-Basic vector (manufactured by Promega) carrying the sequence encoding firefly luciferase but lacking a eukaryotic promoter or enhancer elements, and thereby being cloned.

Deletion fragments having various lengths of the promoter region [-304 to +7] were prepared by PCR method.

The resulting amplified fragment was inserted into the KpnI-XhoI sites of pGL2-Basic vector or pGL2-Promoter vector (manufactured by Promega) carrying SV40 minimal promoter upstream of the sequence encoding firefly luciferase to prepare a reporter promoter for a series of the above deletion mutants.

In order to construct point mutants of the [-139 to -62] and [-65 to -26] regions, synthetic oligonucleotides having appropriate base substitutions were synthesized and annealed, and thereafter the resulting product was ligated to the KpnI-XhoI sites of pGL2-Promoter vector

- 50 -

(manufactured by Promega). The ERSE sequences in the
GRP78 promoter were mutated using Exsite Site-Directed
Mutagenesis Kit manufactured by Stratagene. The resulting
fragments were inserted into the *KpnI-Hind*III sites of
5   pGL3-Basic vector (manufactured by Promega).

A 397-bp fragment of the human GRP94 promoter ([-363
to +34] region) was amplified by PCR method with genomic
DNA of HeLa cells as a template using oligonucleotides as
primers, wherein the oligonucleotides were prepared on the
10   basis of the sequence of a human GRP94 gene disclosed by
Chang et al. [Chang, S.C. et al., *Mol. Cell. Biol.* **9**,
2153-2162 (1989)].

A 511-bp fragment of a human calreticulin promoter
region ([-459 to +52] region) was amplified by PCR method
15   with genomic DNA of HeLa cells as a template using
oligonucleotides as primers, wherein the oligonucleotides
were prepared on the basis of the sequence of a human
calreticulin gene disclosed by McCauliffe et al.
[McCauliffe, D.P. et al., *J. Biol. Chem.* **267**, 2557-2562
20   (1992)].

These two promoters were used in experiment for
disrupting the ERSE sequences. Plasmids purified by
conventional cesium chloride (CsCl) method were used in
transient transfection.

25

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(3)   Transfection Experiments for Transient Expression of

Reporter Plasmids for Isolating ERSE

Transfection was carried out by the conventional

calcium phosphate method described in the above *Molecular*

5   *Cloning*, and the like. The HeLa cells were plated onto a

24-well dish so as to have approximately 10% confluency on

the day before transfection. One microgram of a reporter

plasmid for isolating ERSE and 0.1 µg of a reference

plasmid [pRL-SV40 vector (manufactured by Promega)

10   carrying SV40 enhancer and promoter immediately upstream

of *Renilla* luciferase gene] were mixed at room temperature

in 1 x HEPES buffered saline (composition: 50 mM HEPES,

280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.08) containing 250 mM

$CaCl_2$ to form $CaPO_4$-DNA complex. The cells were incubated

15   with the resulting $CaPO_4$-DNA complex for 16 hours at 37°C,

washed with phosphate buffered saline three times, and

further incubated in a fresh medium.

(4)   Determination of Activity

20   After culturing the transfection cells obtained in

item (3) above for 48 hours, the cells were harvested with

a rubber policeman. The harvested cells were suspended in

100 µl lysis buffer (100 mM potassium phosphate buffer, 1

mM dithiothreitol, pH 7.8). Cells were disrupted by

25   freeze-thawing the resulting cell suspension three times,

- 52 -

whereby obtaining a cell-free extract.

In order to induce endoplasmic reticulum stress response, cells were treated with 2 µg/ml tunicamycin 16 hours prior to harvesting the cells.

5   The firefly luciferase and *Renilla* luciferase activities were determined with 5 µl of cell lysate using the Dual Luciferase Reporter Assay Systems (manufactured by Promega) according to the instruction of the manufacturer. The above activities were measured using

10   luminometer (manufactured by Labsystems, trade name: Luminoskan) within a linear range. The relative luciferase activity was found by normalizing the firefly luciferase activity to the *Renilla* luciferase activity.

15   (5) Identification of ERSE

The endoplasmic reticulum stress response promoter of the mammalian GRP gene was remarked to contain numerous CCAAT motifs. As shown in Figure 3, the structural motif CCAAT-9nt-CCACG is found in the promoters for GRP78, GRP94

20   and calreticulin. As shown in Figures 3 and 4, in these CCAAT sequence and flanking sequences, all of the GRP promoters tested except for FKBP13 have been found to contain multi-copy of similar motifs. As shown in Figure 1, both of CORE and C1 region previously defined in the

25   GRP78 promoter actually contains this motif.

- 53 -

Interestingly, as shown in Figure 2, the mammalian GRP78

promoter comprises continuous repeated sequences, each of

which contains this motif, and it is deduced that the

repeated sequences are caused by the replication during

5   the evolution process. From these findings, it has been

suggested that the motif is specifically contained in the

mammalian unfolded protein response (UPR). Since the

motif is structurally different from UPRE responding to

the yeast UPR, this motif is referred to as an endoplasmic

10   reticulum stress response element (ERSE). In addition,

the ERSE-like sequences are found in vertebrates, plants,

fungi, and the like as shown in Figure 5.

(6)   Involvement in Transcriptional Induction of ERSE

15   Motifs of GRP78, GRP94 and Calreticulin

In order to test whether or not the ERSE motif is

important in the induction of GRP78, GRP94 and

calreticulin, each of the promoters shown on lines 1 to 14

of Figure 6 was ligated to a firefly luciferase gene as a

20   reporter gene, and the ligated product was transfected to

HeLa cells in the same manner as in item (3) above. UPR

was induced by using tunicamycin (hereinafter simply

referred to as "TM"), an inhibitor for N-glycosylation of

a protein.

25   As shown in Figure 6, intact GRP78 promoter has

- 54 -

increased the expression of luciferase in the TM-treated

cells (Figure 6, line 2, closed bar) by 5-fold over that

of the control (line 2, open bar). The extent of

expression was comparable to the one observed in the

5   previous studies [Ting, J. et al., *DNA* 7, 275-286 (1988);

Wooden, S.K. et al., *Mol. Cell Biol.* 11, 5612-5623 (1991)]

(5 to 7 times); and that of the endogenous GRP78 protein

level (5 to 8 times) shown in panel (C) of Figure 19.

In the intact GRP94 promoter and the calreticulin

10   promoter, the luciferase expression was increased by the

TM treatment by 8- and 4-fold, respectively, as shown in

Figure 6, lines 8 and 12. This expression level agrees

with the one previously reported by Ramakrishnan, M. et

al., *DNA Cell Biol.* 14, 373-384 (1995); and Waser, M. et

15   al., *J. Cell Biol.* 138, 547-557 (1997).

In addition, as shown on line 3 of Figure 6, when the

ERSE1 motif was selectively removed from the GRP promoter,

the induction of luciferase was drastically reduced. As

shown on lines 4 to 6 of Figure 6, the induction of the

20   luciferase decreased by the removal of the ERSE1 motif was

completely abolished by further disruption of ERSE2 and/or

ERSE3.

As in the case of GRP78, the disruption of ERSE1 of

the GRP94 promoter (shown on line 9 of Figure 6)

25   drastically decreased the induction, and as shown on line

- 55 -

10, the induction was completely disrupted by simultaneous removal of ERSE1 and ERSE3.

As shown on line 13 of Figure 6, the removal of ERSE3 alone at a distal position of the calreticulin promoter had substantially no effect, but as shown on line 14, the disruption of both ERSE3 and ERSE2 of the calreticulin promoter completely prevented induction (line 14).

As shown in Figure 7, when several deduced ERSEs each carrying some flanking sequences (each of ERSEs of GRP78, GRP94 and calreticulin) were at positions upstream of the heterologous promoter (SV40 minimal promoter), since these ERSEs had markedly induced luciferase activity, there has been suggested that these ERSEs are actually functioning.

In addition, the effects by the orientation of ERSE1 derived from human GRP78 promoter were evaluated. As a result, the same level of activities was exhibited regardless of its orientation. It was suggested from the above results that the ERSE motif is an essential and sufficient cis-acting element for induction of GRP78, GRP94 and calreticulin and perhaps other GRPs.

In the ERSE motif, in order to determine whether or not the essential nucleotide sequence is actually CCAAT(N)$_9$CCACG, a point mutation (transversion) was introduced into each of nucleotides of ERSE1 derived from human GRP78 promoter used in the above experiment.

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        As a result, as shown in Figures 8 and 9, while the

substitutions of other nucleotides had substantially no

effect, some of the substitutions among the nucleotides

[-61 to -57] (Figure 8, lines 7 to 11) or [-46 to -43]

5   (Figure 8, lines 22 to 25) almost completely inhibited the

induction. It was strongly suggested from the above

results that CCAAT (-61 to -57) and CACG (-46 to -43) were

essential for the induction.

        As shown in Figure 3, since the nucleotide C[-47] was

10   well conserved, it was further analyzed in detail. In

this experiment, a [-65 to -38] segment exhibiting a

markedly low response as compared to a [-65 to -42]

segment (Figure 9, line 27) was used. While there were

substantially no effects when C[-47] was changed to A (see

15   line 30; line 21), as shown on lines 31 and 32 of Figure

9, the induction was completely disrupted when C[-47] was

changed to G or T. Therefore, it was shown that C[-47] is

also essential for induction. By contrast, there were

exhibited substantially no effects in the conversion of

20   A[-42] to other nucleotides. Further, since the insertion

of 1 to 3 nucleotides shown on lines 38 to 40 completely

disrupted the induction of luciferase, there was shown

that the distance between CCAAT (-61 to -57) to CCACG (-47

to -43) is important. These results show that the

25   essential sequence of the ERSE1 motif for the human GRP78

- 57 -

promoter is 19 nucleotides stretch [CCAAT(N)$_9$CCACG].

(7)   Response to Other Endoplasmic Reticulum Stress

      Inducer

5   In order to induce experimentally the endoplasmic

reticulum stress response of the mammalian GRP, in

addition to tunicamycin used in all experiments, the cells

were treated with various chemicals including calcium

ionophore A23187 depleting the calcium storage of the

10   endoplasmic reticulum; thapsigargin inhibiting Ca^{2+}-ATPase

of the endoplasmic reticulum and excretion of calcium ions

from the endoplasmic reticulum, or the like. The

endoplasmic reticulum stress response of the human GRP

promoter to A23187 and thapsigargin were tested. The

15   results are shown in Figure 10, wherein lines 1, 5, 9, 13,

17 and 21 indicate the relative activity when no inducer

is added as a control; lines 2, 6, 10, 14, 18 and 22

indicate the relative activity when tunicamycin is added;

lines 3, 7, 11, 15, 19 and 23 indicate the relative

20   activity when calcium ionophore A23187 is added; and lines

4, 8, 12, 16, 20 and 24 indicate the relative activity

when thapsigargin is added.

      Since the transcriptional induction by each treatment

of GRP78 (Figure 10, lines 1 to 4), GRP94 (Figure 10,

25   lines 9 to 12), and calreticulin (Figure 10, lines 17 to

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20) was completely prevented by the disruption of ERSE as

shown on lines 5 to 8, 13 to 16, and 21 to 24 of Figure

10, there was exhibited that ERSE is required for the

induction of GRP not only by tunicamycin but also by other

5   inducers.

Example 2: One-Hybrid Screening for cDNA Encoding ERSE

Binding Protein

       The reporter plasmid for one-hybrid screening was

10   constructed essentially according to the method described

by Mori, K. et al. [*Genes Cells* 1, 803-817 (1996)].

       Six tandem repeats of ERSE1 sequence from the human

GRP78 promoter [5'-CCTTCA<u>CCAAT</u>CGGCGGCCT<u>CCACG</u>ACGG-3'

(SEQ ID NO: 35)] were inserted upstream of the yeast *HIS3*

15   gene ligated to the *IRE1* promoter, while six tandem

repeats of mutant ERSE repeats

[5'-CCTTCAgactaCGGCGGCCTgatgtACGG-3' (SEQ ID NO: 36)] were

inserted upstream of *Escherichia coli lacZ* gene ligated to

the *IRE1* promoter.   The structure of the above reporter

20   plasmid is schematically shown in Figure 11.

       The above reporter plasmids were respectively

linearized at the *NcoI* site present in *URA3* gene, and one

copy thereof was integrated into the *ura3-52* locus of

yeast strain KMY1015 (*MATα leu2-3, 112 ura3-52 his3-Δ200*

25   *trp-Δ901 lys2-801 ire1Δ::TRP1*) [Mori, K. et al., *Genes*

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Cells 1, 803-817 (1996)]. The resulting yeast strain
(hereinafter, referred as KMY1015-ERSE) was used as a host
for eliminating unexpected activation via UPR. The
KMY1015-ERSE was unable to grow in the absence of

5   histidine and expressed low β-galactosidase activity due
to low basal activity of the *IRE1* promoter.

A human lymphocyte cDNA library prepared by using a
multicopy plasmid vector carrying the activation domain of
yeast transcriptional activator Gal4p (GAL4AD) immediately

10   upstream of the cDNA cloning site was kindly provided by
Dr. S.J. Elledge of Baylor College of Medicine through Dr.
N. Hayashi of Kanazawa University.

The ERSE-bound protein, when fused with an active
domain of yeast transcription factor Gal4p (GAL4AD), is

15   expected to activate the transcription of the reporter
gene HIS3 in an ERSE-dependent manner. Therefore,
transformants were prepared using yeast as a host by
introducing the above human lymphocyte cDNA library
provided by Dr. Ellege, and about 4,300,000 transformants

20   were screened. As a result, 8 clones showing strong His[+]
phenotype were obtained. Among the resulting clones,
clones expressing β-galactosidase at a high level despite
the absence of the functional ERSE in the upstream region
were discarded. As a result, there was obtained clone #3,

25   in which the transcription of the reporter gene increases

- 60 -

in an ERSE-dependent manner.

In order to examine the ERSE-dependency of the transcriptional activation by the clone 3, clone 3-GAL4AD fusion protein was expressed together with various

5   reporter plasmids in which intact ERSEs and mutated ERSEs were placed upstream of the lacZ gene ligated with the IRE1 promoter. The results are shown in Figure 12.

As shown in Figure 12, when the intact ERSE was placed upstream of the reporter gene, the β-galactosidase

10   activity level was remarkably increased by the plasmid expressing clone 3-GAL4AD as an effector (line 5) as compared with the β-galactosidase activity level by the control plasmid expressing GAL4AD alone (line 1).

In addition, the increase by the clone 3-GAL4AD, as

15   shown on lines 6 to 8 of Figure 12, was completely disrupted by using mutated ERSE lacking either one or both of CCAAT or CCACG. The results, as shown in Figures 8 and 9, are relevant to the requirement of intact CCAAT and CCACG for transcriptional activation observed in the HeLa

20   cells.

It is unlikely possibility that ectopic expression of a protein derived from clone 3 in yeast cells causes endoplasmic reticulum stress by the translocation of the clone 3-GAL4AD fusion protein into the endoplasmic

25   reticulum lumen, which in turn results in an enhanced

- 61 -

transcription of a reporter gene directly via yeast UPR

pathway in the following two aspects. First, a yeast

strain lacking a sensor molecule Irelp for endoplasmic

reticulum stress was used as a host. Second, ERSE is not

5   functional in yeast as a UPR-specific cis-acting element.

As a result of the nucleotide sequence analysis by a

conventional method, it was elucidated that clone 3

encodes a known transcription factor, ATF6 [Hai, T.W. et

al., *Genes Dev.* 3, 2083-2090 (1989)], a member of the

10   ATF/CREB family containing bZIP motif as a DNA-binding

domain.

Interestingly, as shown in Figure 13, the basic

region of ATF6 shows marked similarity with a basic region

of yeast Haclp. Although the function of ATF6 is not made

15   evident, ATF6 is originally cloned as a partial cDNA

weakly bound to a cAMP response element, and is recently

re-isolated as a protein bound to a serum response factor.

Example 3: Isolation of Entire cDNA Encoding ATF6 and

20   CREB-RP

A portion of 5'-region of mRNA for ATF6 thought to be

lacking in clone #3 was isolated by 5'RACE using HeLa

cells RNA. Here, 5'RACE method employed 5'RACE System

(manufactured by Life Technologies Inc.). An intact ATF6

25   cDNA obtained as described above has a length of 2509 bp,

- 62 -

and a protein having 670 amino acids was encoded (The

accession number for GeneBank Data Base is AB015856). The

deduced amino acid sequence was different from the

sequence reported by Zhu et al. [*Mol. Cell. Biol.* **17,**

5   4957-4966 (1997)] by 4 residues which are deduced to

reflect an allele polymorphism.

An entire cDNA of CREB-RP having a similar molecular

structure and homology in the amino acid sequence is

cloned from HeLa cells RNA by PCR on the basis of the

10   published sequence [Min, J. et al., *Genomics* **30,** 149-156

(1995); Khanna, A. et al., *Biochem. J.* **319,** 81-89 (1996)].

Example 4: Construction of Effector Plasmid

An entire cDNA was inserted into the *Hind*III-*Xho*I

15   sites or *Bam*HI-*Eco*RI sites immediately downstream of the

CMV promoter of pcDNA3.1(+) vector (manufactured by

Invitrogen) to construct an effector plasmid for

expressing ATF6 or CREB-RP, respectively.

20   Example 5: ERSE-Dependency of Transcriptional Activity

(1)   Test for ERSE-Dependency ATF6 on Transcriptional

Activity

In order to examine whether or not ATF6 is involved

in the transcriptional regulation of the GRP gene in

25   mammalian cells, HeLa cells were co-transfected with an

effector plasmid carrying a full-length ATF6 cDNA and a

- 63 -

reporter plasmid resulting from ligation of the firefly

luciferase gene to downstream of the intact GRP promoter

or a mutant GRP promoter, and GRP promoter activity was

evaluated from the luciferase activity. The results are

5   shown in Figure 14.

As indicated by the open bars on lines 1 and 2 in

Figure 14, the ATF6 expression plasmid increased the

luciferase expression from the GRP78 promoter having

intact ERSE sequences by 5-fold over the control level of

10   the aforementioned vector in the absence of TM, but the

expression was not affected from the mutant GRP78 promoter

lacking all of the three ERSE sequences.

In addition, the ATF6 effector plasmid further

increased the β-galactosidase expression from the GRP78

15   promoter having intact ERSE sequences in the presence of

TM, as indicated by the closed bars on lines 1 and 2 in

Figure 14, while the luciferase expression level did not

increase from the mutant GRP78 promoter, as indicated by

the closed bars on lines 3 and 4.

20   Similar results were obtained with other GRP

promoters: GRP94, calreticulin, ERp72 and GRP58. The

results shown on lines 5 through 16 definitely suggested

that enhanced expression of ATF6 is capable of

specifically activating transcription of the GRP genes via

25   ERSE, regardless of the presence or absence of the

- 64 -

endoplasmic reticulum stress.

(2)   Test for ERSE-Dependency on CREB-RP Transcriptional

       Activity

5   As shown in Figure 15, CREB-RP [Min, J. et al.,

Genomics **30**, 149-156 (1995)] shows marked similarity to

ATF6 both in the entire structure and in the deduced amino

acid sequence, especially in its basic region, having 21

out of the 23 residues in common, and the remaining 2

10   being similar basic residues. Also, as shown in Figure

13, CREB-RP possesses the highest similarity among the

known members of the human ATF/CREB family.

          Therefore, with these findings in mind, a test was

carried out to determine whether or not CREB-RP

15   overexpression affects the activity of the GRP promoter.

Interestingly, as indicated by the open bars on lines 1

and 2 in Figure 16, co-transfection of the CREB-RP

expression plasmid hardly affected the reporter expression

from the intact GRP78 promoter in the absence of TM, while

20   the expression was markedly suppressed in the presence of

TM.

          In contrast, as shown on lines 3 and 4, CREB-RP

overexpression did not affect the expression from the

mutant GRP78 promoter in the presence or absence of TM.

25   Because similar results were obtained using the GRP94 or

- 65 -

calreticulin promoter, it has been suggested that CREB-RP

overexpression prevents the endoplasmic reticulum

stress-induced transcription of the GRP gene via an

endogenous trans-activator.

5   It is shown from the results of items (1) and (2) in

Example 5 that the closely related transcription factors

ATF6 and CREB-RP have definitely opposite effects on

induction of target genes, and it was also objected to the

possibility that ATF6 overexpression simply activated GRP

10   transcription indirectly by producing endoplasmic

reticulum stress in HeLa cells.

Example 6: ATF6 Regulation by Endoplasmic Reticulum Stress

        A test was carried out to determine whether or not

15   ATF6 expression is regulated by endoplasmic reticulum

stress on an mRNA level or protein level.

(1)   Northern Blot Hybridization

        Northern blotting was carried out in accordance with

20   the standard method described in *Molecular Cloning: A*

Laboratory Manual, Second Edition [Sambrook, et al.

(1989)]. Concretely, 10 μg of a poly-A$^+$ RNA, prepared from

HeLa cells using oligo(dT) magnetic particles

(manufactured by Dynabeads), was subjected to

25   electrophoresis on 1% agarose gel containing 2.2 M

- 66 -

formaldehyde, transferred to a nylon membrane, and
hybridized with a radiolabeled cDNA specific to either
ATF6 or GAPDH.

As shown in Figure 17, Northern hybridization
5   analysis revealed the presence of a single band for 2.5 kb
ATF6 mRNA in untreated HeLa cells, as reported by Zhu et
al. [Zhu, C. et al., *Mol. Cell Biol.* 17, 4957-4966
(1997)]. In addition, unlike the recently identified
splicing system of an yeast HAC1 mRNA, which is
10   specifically induced by endoplasmic reticulum stress [Cox,
J.S. et al., *Cell* 87, 391-404 (1996); Kawahara, T. et al.,
Mol. Biol. Cell 8, 1845-1862 (1997)], neither an
expression level nor a size of ATF6 mRNA was affected by
TM treatment.

15

(2)   Preparation of Antisera and Immunoblotting

Two types of antisera against ATF6, i.e., anti-B03N
and anti-ATF6 (N21-34), were prepared. The anti-B03N
antiserum was obtained by immunizing a rabbit with a
20   fusion protein of N-terminal portion (6th to 307th
residues) of ATF6 with the *Escherichia coli*
maltose-binding protein, which had been expressed in
Escherichia coli cells and purified. The anti-B03
antiserum thus obtained was treated with CH-Sepharose 4B
25   (manufactured by Amersham Pharmacia Biotech) on which the

soluble proteins of *Escherichia coli*, previously transformed with the maltose-binding protein expression plasmid pMAL-c2 (manufactured by New England Biolab), were immobilized, to yield a flow-through fraction for the

5   purified ATF6 antibody. The anti-ATF6(N21-34) antiserum was obtained by immunizing with the keyhole limpet hemocyanin conjugate-synthesized peptide of 14 N-terminal amino acids (21st to 34th residues) of ATF6. The anti-GRP78 and anti-HSP70 antisera were obtained from

10 Stressgen Biotechnologies Corporation.

     in vitro translation of ATF6 was carried out using ATF6 cDNA and the TNT T7 Quick Coupled Transcription/Translation System (manufactured by Promega).

15   By lysing 1 x 10⁶ HeLa cells in 60 μl of 1 x sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 350 mM dithiothreitol and 0.01% bromophenol blue), a whole cell extract was prepared. This lysate was boiled according to the standard protocol (*Molecular Cloning: A Laboratory*

20 *Manual, Second Edition*), aliquot (2 μl) of which lysate was subjected to electrophoresis on 10% SDS polyacrylamide gel, transferred onto a Hybond ECL filter (manufactured by Amersham Pharmacia Biotech), and reacted with various antisera. A pre-stained SDS-PAGE standard (manufactured

25 by Bio-Rad) was used as a size marker. Using an ECL

- 68 -

Western blotting detection kit (manufactured by Amersham

Pharmacia Biotech), each antigen was detected.

ATF6, previously known to be constitutively expressed

in both HeLa cells and COS cells [Zhu, C. et al., *Mol.*

5   *Cell Biol.* 17, 4957-4966 (1997)], was analyzed by

immunoblotting. The results are shown in Figure 18.

When *in vitro* translation was carried out using a

reticulocyte lysate, ATF6 was detected as a single band of

90 kDa molecular weight by the anti-B03N antiserum, as

10   shown on lane 2 in Figure 18. It was also detected by the

anti-peptide [anti-ATF6(N21-34)] antiserum as a single

band of 90 kDa molecular weight. These molecular weights

were greater than the calculated molecular weight of 74.57

kDa, as in the report of Zhu et al.

15   In contrast, as shown on lane 3, the anti-B03N

antiserum reacted with several kinds of proteins in the

extract from untreated HeLa cells. Of these proteins, the

mobility of the band indicated by the open arrow

corresponded to the band from *in vitro* translated ATF6.

20   When HeLa cells were treated with TM for 4 hours, the 90

kDa band decreased, whereas a new 50 kDa band indicated by

the closed arrow appeared instead, as shown on lane 4. No

other cross-reacted bands were affected.

Importantly, this 50 kDa protein was also detected by

25   the anti-peptide (anti-ATF6 (N21-34)) antiserum (lane 6).

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Furthermore, both the 90 kDa and 50 kDa proteins were

constitutively present in the extract from ATF6

overexpressing cells (lane 8) but not in the extract from

control cells (lane 7), and no other protein bands were

5   produced in excess under the same conditions. This

finding suggests that these two proteins may be actually

encoded by ATF6 cDNA. The 90 kDa and 50 kDa proteins were

designated as p90ATF6 and p50ATF6, respectively.

10   Example 7: Correlation Between p50ATF6 and Intracellular

UPR Activity

        In order to determine whether or not the appearance

of p50ATF6 is associated with the intracellular UPR

activity, stress-induced p50ATF6 appearance and GRP78

15   expression were monitored with the passage of time by

immunoblotting. The results are shown in Figure 19.

        As shown in Figure 19, increase in GRP78 was detected

8 hours after the TM treatment, whereas increase in

p50ATF6 became detectable within 2 hours, reaching a peak

20   at 4 to 8 hours, then showing a decrease. Similarly,

p50ATF6 appeared in advance of GRP78 induction even in

cells treated with A23187 or thapsigargin (indicated by Tg

in the figure).

        However, in cells subjected to heat shock, p50ATF6

25   was hardly detected, even though the definite induction of

- 70 -

HSP70 was found.

These results strongly suggest that the conversion of p90ATF6 to p50ATF6 is an important regulation process in mammalian UPR.

Example 8: Conversion of p90ATF6 to p50ATF6 by Endoplasmic Reticulum Stress

In order to determine whether or not p90ATF6 is directly converted to p50ATF6 by endoplasmic reticulum stress, quantitative changes in p90ATF6, p50ATF6 and the target protein GRP78 in TM-treated HeLa cells were evaluated with the passage of time by immunoblotting using the purified ATF6 antibody.

As shown in Figure 20, p50ATF6 appeared at 2 hours after the TM treatment and continued to be detected until 4 hours (lanes 3 to 5). On the other hand, p90ATF6 decreased with the passage of time after the TM treatment, showing clear reverse correlation with the increase in p50ATF6 (lanes 1 to 6). In addition, increased GRP78 expression was observed from 8 hours after the TM treatment. These results suggest that ATF6 is synthesized as a precursor protein (p90ATF6) and specifically converted to a mature protein (50ATF6) by endoplasmic reticulum stress.

In the experiment with respect to the passage of time

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shown in Figure 20, in addition to p50ATF6, a new band
with a slightly faster mobility than that of p90ATF6 was
observed in TM-treated HeLa cells, whereby suggesting the
possibility that p90ATF6 was modified by glycosylation and
5   hence associated with the endoplasmic reticulum. In fact,
when unstressed HeLa cells were analyzed by the indirect
immunofluorescence method using the anti-B03N antibody,
fine reticular stained images were observed around the
nucleus. This staining pattern was the same as that
10   observed using an anti-KDEL antibody (10C3; manufactured
by Stressgen) which recognized the principal endoplasmic
reticulum chaperones of GRP78 and GRP94. Furthermore, as
a result of the computer-aided hydropathy analysis, it was
found that as shown in Figure 21, there exists a
15   hydrophobic region having 21 amino acids, which is long
enough to penetrate the membrane once. These results
strongly support the idea that p90ATF6 is a membrane
protein in the endoplasmic reticulum.

Next, a homogenate of HeLa cells treated with or
20   without TM for 4 hours was fractionated by centrifugation
to evaluate the localization of p90ATF6 and p50ATF6
(Figure 22). By the first low-speed centrifugation, the
majority of p90ATF6 was recovered in the soluble fraction
(lane 5), whereas p50ATF6 was fractionated selectively in
25   the nuclear fraction (lane 4), similarly to the nuclear

- 72 -

protein lamin B. By the subsequent high-speed
centrifugation, whole p90ATF6 was collected in the
membrane fraction (lane 9). Although the distribution
pattern of p90ATF6 was substantially identical to that of

5   calnexin, an endoplasmic reticulum transmembrane
chaperone, it completely differed from that of lamin B or
the cytosolic protein HSP70. Lamin B and HSP70 were
detected using an anti-lamin B antibody (manufactured by
Santa Cruz) and an anti-HSP70 antibody (C92F3A-5;

10   manufactured by Stressgen), respectively.

Example 9: Topology of p90ATF6

    A test was carried out in order to determine whether
p90ATF6 is a peripheral or integral membrane protein. The

15   soluble fraction obtained by low-speed centrifugation was
subjected to various treatments, and subsequently
fractionated by high-speed centrifugation. Immunoblotting
was carried out using the purified ATF6 antibody. As a
result, as shown in Figure 23, in a treatment with 0.5 M

20   NaCl or 0.1 M Na_2CO_3 (pH 11), in which peripheral membrane
proteins were extracted, p90ATF6 was not released from the
membrane as well as the integral membrane protein
calnexin. In contrast, p90ATF6 and calnexin were both
released in the soluble fraction by a detergent, such as

25   1% SDS or 1% sodium deoxycholate (DOC). 1% Triton X-100

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may cause aggregation of p90ATF6.

Next, the orientation of p90ATF6 to the membrane was examined by trypsin treatment (Figure 24). Trypsin digestion was monitored using calnexin, a type I membrane protein, as a control, and using an antibody (manufactured by Stressgen) recognizing the calnexin N-terminal region in the endoplasmic reticulum lumen (calnexin-N) or the calnexin C-terminal region in the cytosol (calnexin-C). At the trypsin concentrations reducing the amount of full-length calnexin, the appearances of indigestible calnexin fragment were not observed with the anti-calnexin C-antibody (lanes 11 and 12), while the appearance of a fragment of a size corresponding to the calnexin N-terminal region was observed with the anti-calnexin N antibody (lanes 7 and 8). This result demonstrates that the region of the lumenal side was resistant to trypsin digestion as expected.

Under these experimental conditions, p90ATF6 disappeared at the lowest trypsin concentration examined, and no fragment corresponding to 50 kDa molecular weight could be detected by the anti-ATF6 antibody recognizing the N-terminal region of ATF6 (lane 2). This result strongly suggests that p90ATF6 is a type II membrane protein with its N-terminal region oriented toward the cytosol.

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Example 10: Intracellular Localization of p50ATF6

In order to determine whether or not the
intracellular localization status is altered by converting
p90ATF6 to p50ATF6, a test was carried out by an indirect
5   immunofluorescence method using HeLa cells transformed
with the ATF6 expression plasmid. Plasmid pCGN-ATF6 [Zhu
et al., *Mol. Cell. Biol.* **17**, 4957-4966 (1997)] capable of
expressing full-length ATF6 was obtained from Dr. Prywes
of Columbia University (hereinafter referred to as
10   pCGN-ATF6(670)). This plasmid was prepared by inserting
ATF6 cDNA to the *Xba*I site of the expression vector pCGN
for animal cells, and expressed ATF6 with the influenza
virus hemagglutinin (HA) epitope bound to the N-terminus
thereof under the control of the cytomegalovirus promoter.
15   The plasmids pCGN-ATF6(402), pCGN-ATF6(373),
pCGN-ATF6(366) and pCGN-ATF6(330), all of which cause the
expression of an N-terminal fragment of ATF6, were
constructed by preparing the regions encoding amino acids
1st to 402nd, 1st to 373rd, 1st to 366th and 1st to 330th,
20   respectively, by PCR, and inserting them, together with
the stop codon (TAG), in the *Xba*I site of pCGN.

The structures of the ATF6 and various mutants
expressed from these plasmids are schematically shown in
Figure 25. ATF(402) lacks the majority of the C-terminal
25   lumenal domain but retains the transmembrane domain

- 75 -

(V^{334}-L^{398}), whereas the three mutants ATF6(373), ATF6(366) and ATF6(330) lack both the lumenal domain and the transmembrane domain. ATF6(373) comprises the basic region (R^{308}-R^{330}) and the leucine zipper (L^{334}-L^{369}) in their

5   entireties. ATF6(366) comprises the entire basic region and the majority of the leucine zipper region. ATF(330) comprises the basic region but completely lacks the leucine zipper region. All these proteins have the HA epitope bound to the N-terminus thereof.

10   Next, the HeLa cells were transiently transformed with expression plasmids for these ATF6s and various mutants, respectively, and the resulting transformant was analyzed by the indirect immunofluorescence method using an anti-HA epitope antibody (Y11; manufactured by Santa

15   Cruz). The expression of ATF6(670) and ATF6(402), both of which have the transmembrane domain, was found to be localized in the endoplasmic reticulum, showing the same staining pattern as that of the fluorescent staining using an anti-KDEL antibody.

20   On the other hand, in cells expressing ATF6(373), ATF6(366) or ATF6(330), which are mutant ATF6 members lacking both the lumenal domain and the transmembrane domain, the nucleus was clearly stained with the anti-HA antibody. These results strongly suggested that p90ATF6

25   is embedded in the endoplasmic reticulum membrane, whereas

- 76 -

p50ATF6 is cleaved on the N-terminal side of the transmembrane domain and localized in the nucleus.

Example 11: Deduction of Cleavage Site in p50ATF6
5   Production

        In order to deduce the cleavage site involved in p50ATF6 production in response to endoplasmic reticulum stress, the HeLa cells transfected with various C-terminal deletion mutants prepared in Example 10 were analyzed by
10   immunoblotting. The results are shown in Figure 26. In the cells transfected with pCGN-ATF6(670), the two protein bands (lane 2) detected at a position corresponding to about 50 kDa molecular weight served as appropriate molecular weight markers for p50ATF6. These proteins are
15   assumed to be p50ATF6 produced by proteolysis constitutively activated by overexpression of HA-labeled ATF6(670) in the transfected cells. The bands of fast mobility are assumed to have resulted from the loss of the HA label. It is found from the mobility comparison of
20   various C-terminal deletion mutants on SDS-PAGE that the size of p50ATF6 approximates that of ATF6(373), suggesting that when cells are subjected to endoplasmic reticulum stress, p90ATF6 is cleaved between bZIP and the transmembrane domain to produce p50ATF6.

25

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Example 12: Transcription-Activating Domain of ATF6

        Various PCR-amplified regions of ATF6 were inserted

into the *Xba*I site of the plasmid pBIND (manufactured by

Promega), which expresses the DNA-binding domain (amino

5   acids 1st to 147th, referred to as "GAL4DB") of the yeast

transcription factor Gal4p under the control of the

cytomegalovirus promoter, to yield plasmids for expressing

fusion proteins of the ATF6 fragments with GAL4DB. These

expression plasmids were transiently introduced into HeLa

10   cells together with the reporter plasmid pG5luc

(manufactured by Promega) containing five Gal4p binding

sites just upstream of the adenovirus major late promoter

to determine their ability of activating the transcription

of the luciferase gene. As shown in Figure 27, the

15   transcription-activating ability was mapped to an

N-terminal 150-amino acid region (lines 6 and 9 to 12),

especially demonstrating the great contribution of the 43

N-terminal amino acids (lines 6 to 8). The presence of a

transcription-activating domain at the N-terminus ensures

20   the function of p50ATF6 as a transcription factor in the

nucleus.

Example 13: Transcriptional Activity of C-terminus

Deletion Mutants of ATF6

25   Using HeLa cells co-transfected with the reporter

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plasmid pGL3-G78(-132) [see Example 1 (Figure 6, line 2)]
resulting from ligation of the firefly luciferase gene to
downstream of the human GRP78 promoter ([-132 to +7],
numbered on the basis of the translation initiation point

5   taken as +1) and the expression plasmid for the ATF6
C-terminal deletion mutant of Example 10, the effects of
each of the overexpressed C-terminal deletion mutants of
ATF6 on ERSE-mediated transcriptional activation were
evaluated.

10   As a result, it was found that when the full-length
ATF6, namely ATF(670), is transiently overexpressed using
pCGN-ATF6(670), the transcription of the luciferase gene
from the GRP78 promoter having ERSE, is constitutively
activated in the absence of endoplasmic reticulum stress

15   (Figure 28, line 2), as in the results obtained in Example
5 (Figure 14). The higher relative luciferase activity in
comparison with the level obtained in Example 5 (Figure
14) was attributed to the expression of more ATF6 in the
cells transfected with pCGN-ATF6 than in those transfected

20   with pcDNA-ATF6. Further, the constitutive activation of
transcription of the luciferase gene can be well explained
by the fact that a p50ATF6-like protein was constitutively
produced in the aforementioned cells transfected with
pCGN-ATF6(670) (see Figure 26, lane 2). Similarly, the

25   reporter luciferase activity was also constitutively

- 79 -

enhanced by overexpression of endoplasmic

reticulum-localized ATF6(402) [open bar on line 3 in

Figure 28].

    The results as shown by the open bars on lines 4 and 5

5   and the open bars on lines 2 and 3 in Figure 28 demonstrate

that increased luciferase activity by 5-fold or more over

that obtained with the endoplasmic reticulum-localized ATF6

ATF(670) or ATF(402) is obtained by overexpressing the

nucleus-localized ATF6 mutants ATF6(373) and ATF6(366).

10 These results agree with the fact that the nuclear protein

p50ATF6 shows the activated form of ATF6.

    On the other hand, another nucleus-localized

ATF6(330) showed markedly lower activity than ATF6(373) or

ATF(366), despite having nearly the same expression level

15 (Figure 26), demonstrating the importance of the leucine

zipper in the transcriptional activity of ATF6 (compare

line 6 with lines 4 and 5 in Figure 28).

Example 14: Transcription-Activating Domain Deletion

20 Mutants of ATF6

    Each of pCGN-ATF6(151-670), pCGN-ATF6(151-402),

pCGN-ATF6(151-373), pCGN-ATF6(151-366) and

pCGN-ATF6(151-330), which expressed mutant ATF6 lacking a

region of amino acids 1st to 150th from the N-terminal of

25 ATF6 or the mutants prepared in Example 10, was

- 80 -

constructed by preparing the regions encoding amino acids

151st to 670th, 151st to 402nd, 151st to 373rd, 151st to

366th and 151st to 330th, respectively, by PCR, and

inserting each region, together with the stop codon, in

5   the XbaI site of pCGN (see Figure 25).

Being expressed from these plasmids, each of

ATF6(151-670), ATF6(151-402), ATF6(151-373), ATF6(151-366)

and ATF6(151-330) resulted from the deletion of the

N-terminal 150 amino acids (M^1-L^{150}) from ATF6(670),

10   ATF6(402), ATF6(373), ATF6(366) and ATF6(330),

respectively.

In the same manner as in Example 10, HeLa cells were

transiently transformed with each expression plasmid for

an ATF6 transcription-activating domain deletion mutant,

15   and the intracellular localization of the ATF6

transcription-activating domain deletion mutant expressed

was evaluated by the indirect immunofluorescence method

using an anti-HA epitope antibody (Y11; manufactured by

Santa Cruz). As a result, ATF6(151-670) and ATF6(151-402)

20   were found to be localized in endoplasmic reticulum, as

were the corresponding ATF6(670) and ATF6(402),

respectively. In addition, ATF6(151-373), ATF6(151-366)

and ATF6(151-330) were all localized in the nucleus, as

were ATF6(373), ATF6(366) and ATF6(330).

25   Next, in the same manner as in Example 13, the effect

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of the overexpression of each ATF6 transcription-
activating domain deletion mutant on ERSE-mediated
transcriptional activation was evaluated by
co-transfection with the reporter plasmid pGL3-G78(-132).

5   As a result, as shown on lines 7 to 11 in Figure 28,
all ATF6 transcription-activating domain deletion mutants
strongly suppressed the expression of the reporter gene to
a level equal to or less than that when the control pCGN
vector (line 1) is used. ATF6(151-373), ATF6(151-366) and

10   ATF6(151-330) were shown to act dominant-negatively on
endogenous p50ATF6 in the nucleus. On the other hand,
ATF6(151-670) and ATF6(151-402) were assumed to act
dominant-negatively on endogenous p50ATF6 by inducing
endoplasmic reticulum stress by their overexpression in

15   the endoplasmic reticulum, followed by migration into the
nucleus of the resulting p50ATF6 lacking the
transcription-activating domain.

Example 15: Conversion of p110CREB-RP to p60CREB-RP by

20   Endoplasmic Reticulum Stress
       In order to elucidate whether or not a full-length
CREB-RP (p110CREB-RP) is converted to p60CREB-RP by
endoplasmic reticulum stress, quantitative changes in
p110CREB-RP and p60CREB-RP in TM-treated HeLa cells were

25   evaluated with the passage of time by immunoblotting using

- 82 -

a purified CREB-RP antibody.   The CREB-RP antibody was

prepared by immunizing a rabbit with a fusion protein of a

region of amino acids 1st to 307th of CREB-RP with GST,

expressed in *Escherichia coli,* and used after absorption

5   with GST and bacterial proteins and affinity purification

with the GST-CREB-RP fusion protein.

When the conversion of p90ATF6 to p50ATF6 and the

amount of the target protein GRP78 were also examined in

the same manner as in Example 8, p60CREB-RP was found to

10   appear 2 hours after the TM treatment, and, unlike

p50ATF6, to be persistently expressed thereafter, as shown

in Figure 29.

Example 16: Activated Form of CREB-RP

15   From the CREB-RP expression plasmid prepared in

Example 4, a DNA fragment encoding a region of amino acids

1st to 389th of CREB-RP was prepared by PCR, and inserted

into the *Hind*III site of the pcDNA3.1(+) vector.   Being

expressed from this plasmid, CREB-RP(1-389) comprises the

20   N-terminus through the basic region and the leucine zipper

region of CREB-RP, corresponding to p60CREB-RP as

converted from p110CREB-RP by endoplasmic reticulum

stress.

The CREB-RP(1-389) expression plasmid and a reporter

25   plasmid resulting from ligation of the luciferase gene to

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downstream of the GRP78 promoter or mutant GRP78 promoter

were introduced into the HeLa cells to evaluate the effect

of CREB-RP(1-389) overexpression on ERSE-mediated

transcriptional activation.

5   As shown in Figure 30, the overexpression of

CREB-RP(1-389), which is assumed to correspond to

p60CREB-RP, constitutively activated the transcription, in

the same manner as in p50ATF6. Because this

transcriptional activation is not observed when mutant

10   ERSE is used, it is found to be ERSE-dependent.

Example 17: Suppressive Form of CREB-RP

        A DNA fragment encoding a region of amino acids 308th

to 386th of CREB-RP, in which Met was added to the

15   N-terminus thereof, was prepared from the CREB-RP

expression plasmid prepared in Example 4 by PCR, and the

DNA fragment was inserted into the HindIII site of the

pcDNA3.1(+) vector. Being expressed from this plasmid,

CREB-RP(308-386) does not contain the transcription-

20   activating domain present in the N-terminal region of

CREB-RP but only selectively comprises the basic region

and the leucine zipper region.

        In the same manner as in Example 16, the effect of

CREB-RP(308-386) overexpression on ERSE-mediated

25   transcriptional activation was evaluated. As shown in

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Figure 31, the ERSE-dependent transcriptional induction by
the TM treatment was strongly suppressed. The results
demonstrate that CREB-RP(308-386) acts dominant-negatively
on the endogenous endoplasmic reticulum stress
5   transcription factor.

       In addition, in the same manner as in Example 16, the
effect of overexpression of CREB-RP(81-389) or
CREB-RP(151-389) on ERSE-mediated transcriptional
activation was evaluated. Here, each of CREB-RP(81-389)
10   and CREB-RP(151-389) lacks transcription-activating
domain. As shown in Figure 32, the ERSE-dependent
transcriptional induction by the TM treatment was strongly
suppressed. The results demonstrate that each of
CREB-RP(81-389) and CREB-RP(151-389) dominant-negatively
15   acts on the endogenous endoplasmic reticulum stress
transcription factor.

SEQUENCE LISTING FREE-TEXT

       In SEQ ID NO: 1, "n" is A or C or G or T. The
20   sequence as shown in SEQ ID NO: 1 is the ERSE1 consensus
sequence.

       In SEQ ID NO: 2, "n" is A or C or G or T. The
sequence as shown in SEQ ID NO: 2 is the ERSE2 consensus
sequence.

25   In SEQ ID NO: 3, "n" is A or C or G or T. The

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sequence as shown in SEQ ID NO: 3 is the ERSE3 consensus
sequence.

INDUSTRIAL APPLICABILITY

5   The endoplasmic reticulum stress transcription factor
is capable of regulating increase or decrease of
expression of endoplasmic reticulum chaperone genes.
According to the method for controlling expression of
endoplasmic reticulum chaperones of the present invention,

10   the increase or decrease of expression of endoplasmic
reticulum chaperone genes can be regulated. Further,
treatment or prophylaxis of cancers, arteriosclerosis,
cystic fibrosis, ischemic diseases, wounds or ulcers is
made possible by the method for controlling expression of

15   endoplasmic reticulum chaperones of the present invention.
Moreover, by applying the method for controlling
expression mentioned above to expression of a foreign
useful protein, there are exhibited excellent effects that
the foreign useful protein retains correct conformation,

20   and that the foreign protein can be expressed at a high
level.

EQUIVALENT

           Those skilled in the art will recognize, or be able

25   to ascertain using simple routine experimentation, many

equivalents to the specific embodiments of the invention described in the present specification. Such equivalents are intended to be encompassed in the scope of the following claims.

5

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CLAIMS

1.   An endoplasmic reticulum stress transcription factor
capable of regulating transcription-inducing activity,
wherein said transcription-inducing activity is exhibited
by an element having the nucleotide sequence as shown in
SEQ ID NO: 1 or an element having a nucleotide sequence
resulting from substitution of 1 to 3 bases with other
kind of bases in the nucleotide sequence as shown in SEQ
ID NO: 1.

2.   The endoplasmic reticulum stress transcription factor
according to claim 1, wherein said endoplasmic reticulum
stress transcription factor is bZIP transcription factor.

3.   The endoplasmic reticulum stress transcription factor
according to claim 2, wherein said bZIP transcription
factor is ATF6 (SEQ ID NO: 32) or CREB-RP (SEQ ID NO: 34).

4.   The endoplasmic reticulum stress transcription factor
according to claim 2, comprising a polypeptide which can
be encoded by a nucleic acid selected from the group
consisting of:
(A) a nucleic acid having the nucleotide sequence as shown
in SEQ ID NO: 31;

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(B) a nucleic acid having the nucleotide sequence as shown
in SEQ ID NO: 33;

(C) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of one or

5   more bases in the nucleotide sequence of the nucleic acid
(A) or (B); and

(D) a nucleic acid capable of hybridizing under stringent
condition to the strand complementary to any one of
nucleic acids (A) to (C).

10

5.   The endoplasmic reticulum stress transcription factor
according to claim 2, wherein said bZIP transcription
factor is an activated form of ATF6.

15 6.   The endoplasmic reticulum stress transcription factor
according to claim 5, wherein said activated form of ATF6
is p50ATF6 resulting from processing by endoplasmic
reticulum stress.

20 7.   The endoplasmic reticulum stress transcription factor
according to claim 5, wherein said activated form of ATF6
is a polypeptide obtainable by expression of DNA encoding
p50ATF6.

25 8.   The endoplasmic reticulum stress transcription factor

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according to claim 5, wherein said activated form of ATF6

is a polypeptide comprising an entire or partial portion

of a region of 1st to 373th amino acids or a region of 1st

to 366th amino acids in ATF6.

5

9.   The endoplasmic reticulum stress transcription factor

according to claim 2, wherein said bZIP transcription

factor is an activated form of CREB-RP.

10   10.   The endoplasmic reticulum stress transcription factor

according to claim 9, wherein said activated form of

CREB-RP is p60CREB-RP resulting from processing by

endoplasmic reticulum stress.

15   11.   The endoplasmic reticulum stress transcription factor

according to claim 9, wherein said activated form of

CREB-RP is a polypeptide obtainable by expression of DNA

encoding p60CREB-RP.

20   12.   The endoplasmic reticulum stress transcription factor

according to claim 9, wherein said activated form of

CREB-RP is a polypeptide comprising an entire or partial

portion of a region of 1st to 389th amino acids in

CREB-RP.

25

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13.   The endoplasmic reticulum stress transcription factor according to claim 2, wherein said bZIP transcription factor is a suppressive form of ATF6.

5   14.   The endoplasmic reticulum stress transcription factor according to claim 13, wherein said suppressive form of ATF6 is a polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 150th amino acids in ATF6.

10

15.   The endoplasmic reticulum stress transcription factor according to claim 13, wherein said suppressive form of ATF6 is a polypeptide resulting from disrupion of an entire or partial portion of a region of 1st to 150th

15   amino acids in the activated form of ATF6 of any one of claims 5 to 8.

16.   The endoplasmic reticulum stress transcription factor according to claim 2, wherein said bZIP transcription

20   factor is a suppressive form of CREB-RP.

17.   The endoplasmic reticulum stress transcription factor according to claim 16, wherein said suppressive form of CREB-RP is a polypeptide resulting from disruption of an

25   entire or partial portion of a region of 1st to 307th

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amino acids in CREB-RP.

18. The endoplasmic reticulum stress transcription factor
according to claim 16, wherein said suppressive form of
5   CREB-RP is a polypeptide resulting from disruption of an
entire or partial portion of a region of 1st to 307th
amino acids in the activated form of CREB-RP of any one of
claims 9 to 12.

10   19. A method for controlling expression of an endoplasmic
reticulum chaperone, comprising expressing the endoplasmic
reticulum stress transcription factor as defined in any
one of claims 1 to 18.

15   20. The method for controlling expression according to
claim 19, comprising regulating an expression level of an
endoplasmic reticulum stress transcription factor in a
cell.

20   21. The method for controlling expression according to
claim 19 or 20, comprising positively or negatively
regulating expression of an endoplasmic reticulum
chaperone gene by selection of an endoplasmic reticulum
stress transcription factor to be expressed.

25

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22. A method for expressing a foreign protein, comprising positively regulating expression of an endoplasmic reticulum chaperone gene by the method as defined in any one of claims 19 to 21.

5

23. A nucleic acid encoding the activated form of ATF6 as defined in any one of claims 6 to 8, or the complementary strand thereto.

10   24. The nucleic acid according to claim 16, wherein said nucleic acid is selected from the group consisting of:
(a) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 373 in SEQ ID NO: 32;

15   (b) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 366 in SEQ ID NO: 32;
(c) a nucleic acid having a nucleotide sequence as shown in base numbers: 69 to 1187 in SEQ ID NO: 31;

20   (d) a nucleic acid having a nucleotide sequence as shown in base numbers: 69 to 1166 in SEQ ID NO: 31;
(e) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (a) to (d); and

25   (f) a nucleic acid capable of hybridizing to a strand

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complementary to the nucleic acid of any one of (a) to (e)

under stringent condition,

or the complementary strand thereto.

5   25.   A nucleic acid encoding an activated form of CREB-RP

as defined in any one of claims 10 to 12, or the

complementary strand thereto.

26.   The nucleic acid according to claim 25, wherein the

10   nucleic acid is selected from the group consisting of:

(g) a nucleic acid having a nucleotide sequence encoding

an amino acid sequence as shown in amino acid numbers: 1

to 389 in SEQ ID NO: 34;

(h) a nucleic acid having a nucleotide sequence as shown

15   in base numbers: 47 to 1213 in SEQ ID NO: 33;

(i) a nucleic acid having a nucleotide sequence having

substitution, deletion, addition or insertion of at least

one base in the nucleic acid of any one of (g) to (h); and

(j) a nucleic acid capable of hybridizing to the strand

20   complementary to the nucleic acid of any one of (g) to (i)

under stringent condition,

or the complementary strand thereto.

27.   A nucleic acid encoding the suppressive form of ATF6

25   as defined in claims 14 or 15, or the complementary strand

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thereto.

28. The nucleic acid according to claim 27, wherein the
nucleic acid is selected from the group consisting of:

5   (k) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 151
to 670 in SEQ ID NO: 32;

(l) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 151

10   to 373 in SEQ ID NO: 32;

(m) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 151
to 366 in SEQ ID NO: 32;

(n) a nucleic acid having a nucleotide sequence as shown

15   in base numbers: 519 to 2078 in SEQ ID NO: 31;

(o) a nucleic acid having a nucleotide sequence as shown
in base numbers: 519 to 1187 in SEQ ID NO: 31;

(p) a nucleic acid having a nucleotide sequence as shown
in base numbers: 519 to 1166 in SEQ ID NO: 31;

20   (q) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of at least
one base in the nucleic acid of any one of (k) to (p); and
(r) a nucleic acid capable of hybridizing to the strand
complementary to the nucleic acid of any one of (k) to (q)

25   under stringent condition,

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or the complementary strand thereto.

29.   A nucleic acid encoding the suppressive form of
CREB-RP as defined in claim 17 or 18.

5

30.   The nucleic acid according to claim 29, wherein said
nucleic acid is selected from the group consisting of:
(s) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 308
10   to 386 in SEQ ID NO: 34;
(t) a nucleic acid having a nucleotide sequence as shown
in base numbers: 968 to 1204 in SEQ ID NO: 33;
(u) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 151
15   to 389 in SEQ ID NO: 34;
(v) a nucleic acid having a nucleotide sequence as shown
in base numbers: 497 to 1213 in SEQ ID NO: 33;
(w) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 81
20   to 389 in SEQ ID NO: 34;
(x) a nucleic acid having a nucleotide sequence as shown
in base numbers: 287 to 1213 in SEQ ID NO: 33;
(y) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of at least
25   one base in the nucleic acid of any one of (s) to (x); and

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(z) a nucleic acid capable of hybridizing to the strand

complementary to the nucleic acid of any one of (s) to (y)

under stringent conditions,

or the complementary strand thereto.

F I G. 1
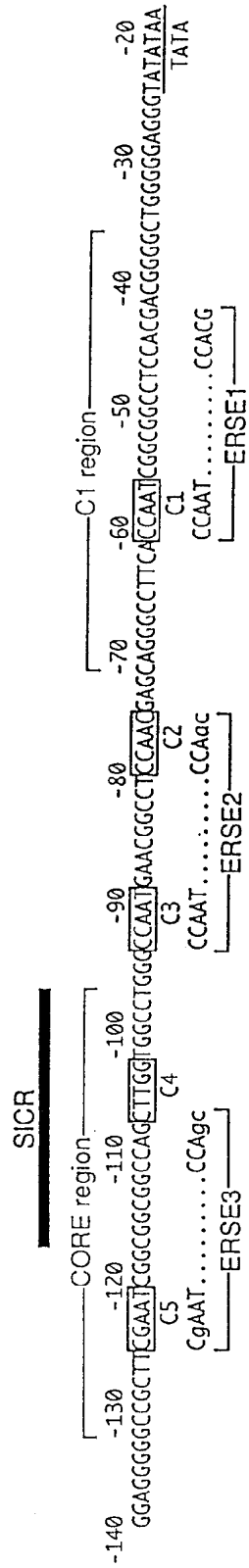
```
                              |-------ERSE------|
                              CCAAT........CCACG

(-139)   GAGGGGGCCGCTTCGAATCGGCGGCGGCCAGC            (-108)
(-107)   TTGGTGGCGTGGGCCAATGAACGGCCTCCAACG           (-75)
(-74)    AGCAGGGCCTTCACCAATCGGCGGCCTCCACGA           (-42)
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F I G .   2

```
GRP78 ERSE1 (human)      CCAATCGGCGGCCTCCACG
      ERSE1 (murine)     CCAATCGGAGGCCTCCACG
      ERSE1 (rat)        CCAATCGGAGGCCTCCACG
GRP94 ERSE1 (human)      CCAATCGCGCCGCACCACG
      ERSE1 (chicken)    CCAATGGGAGCGCACCACG
      ERSE3 (human)      CCAATCGGAAGGAGCCACG
      ERSE3 (chicken)    CCAATCGACGCCGGCCACG
CRT   ERSE3 (human)      CCAATGATGGTCGACCACG
      ERSE3 (murine)     CCAATGAGGGTCGACCACG


GRP78 ERSE2 (human)      CCAATGAACGGCCTCCAAC
      ERSE2 (murine)     CCAATCAGCGGCCTCCAAC
      ERSE2 (rat)        CCAACCAGCGGCCTCCAAC
      ERSE3 (human)      CGAATCGGCGGCGGCCAGC
      ERSE3 (murine)     CGAATCGGCAGCAGCCAGC
      ERSE3 (rat)        CGAATCGGCAGCGGCCAGC
GRP94 ERSE2 (human)      CCAATCGGAGCTGTCCAGG
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      ERSE4 (human)      CCAATCAAATGGCTCCGCG
CRT   ERSE1 (human)      CCAATGACAAAGTGGCAGG
      ERSE2 (human)      CCAATAGAAATCGGCCATC
      ERSE2 (murine)     CCAATAGAAATCAGCCATC
ERp72 ERSE1 (murine)     CCAATCAGAAGGGGGCACC
      ERSE2 (murine)     CCAATCACGGGCTGCCACT
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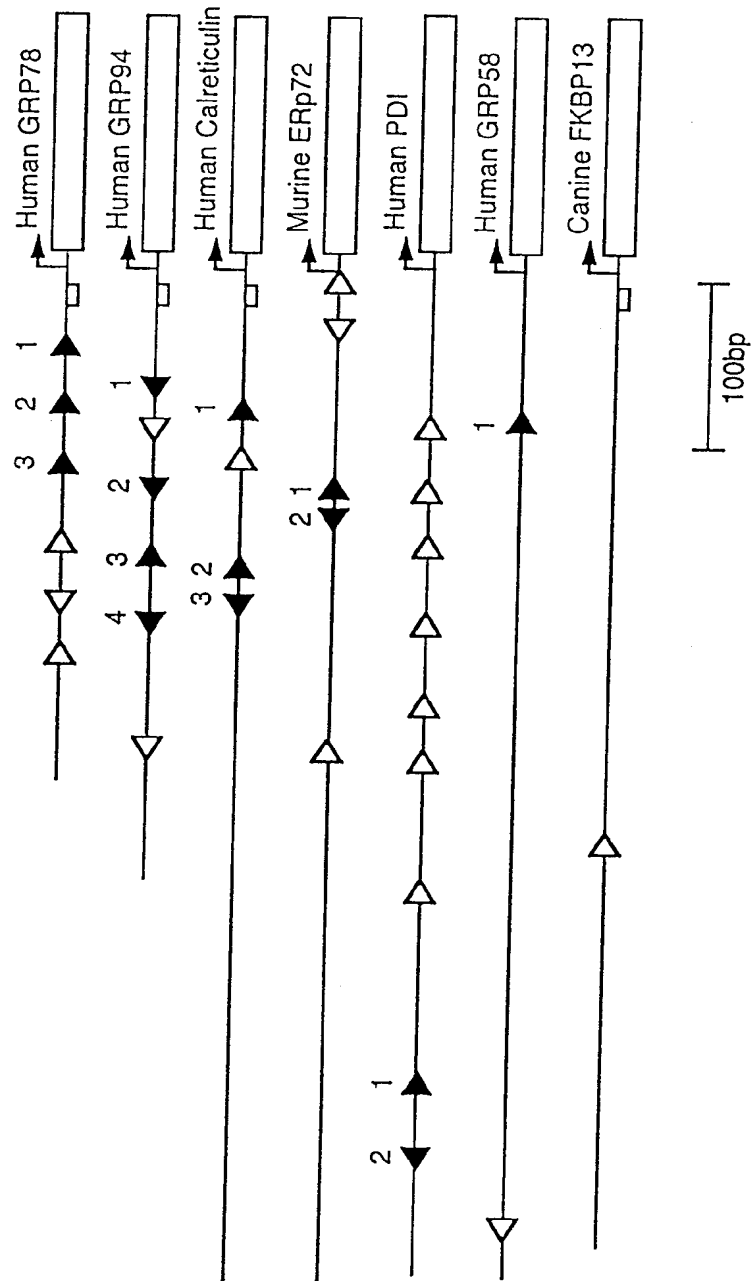
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F I G. 6

F I G . 7

F I G . 8

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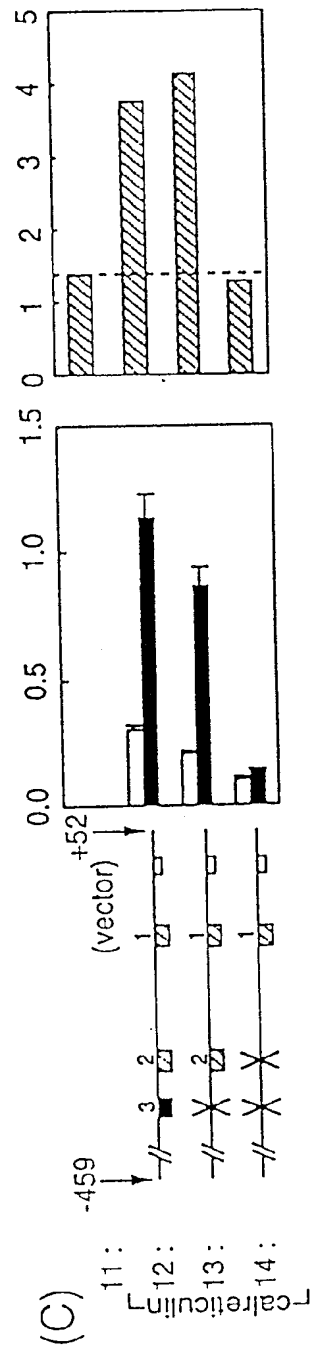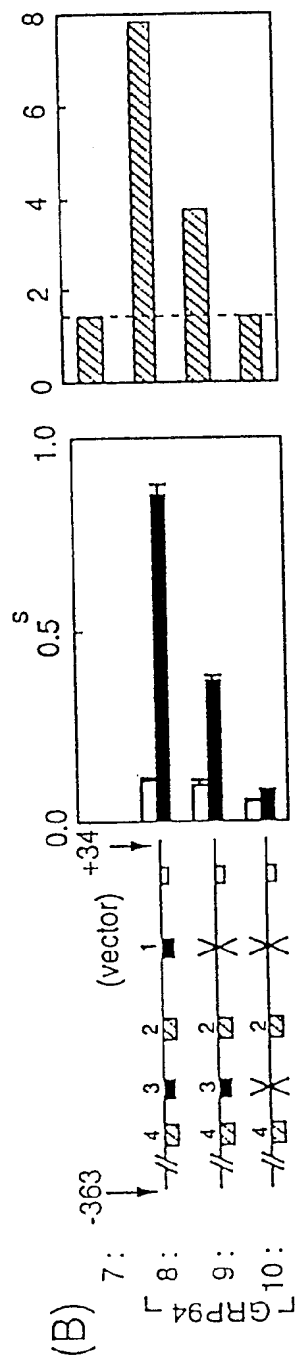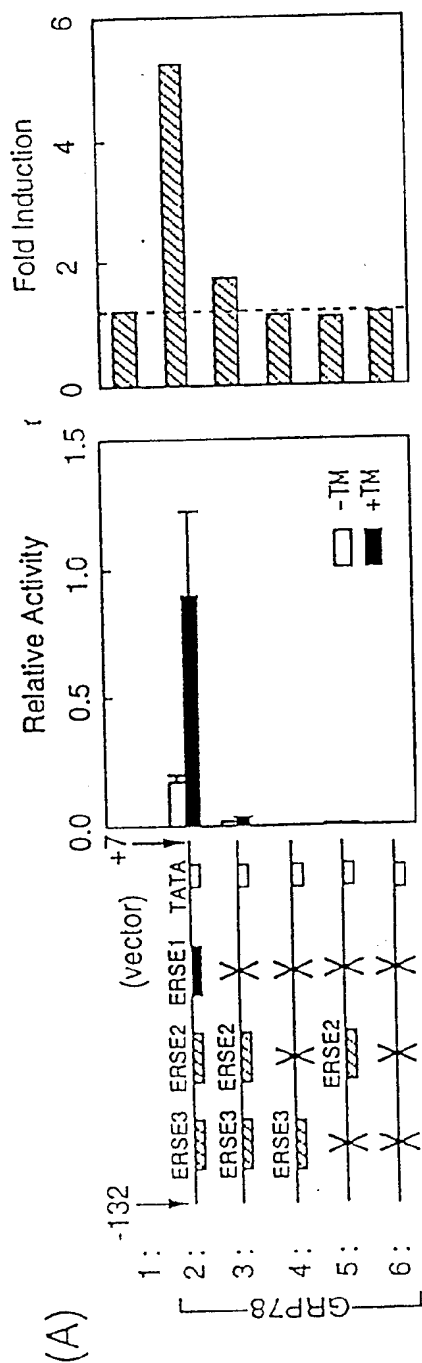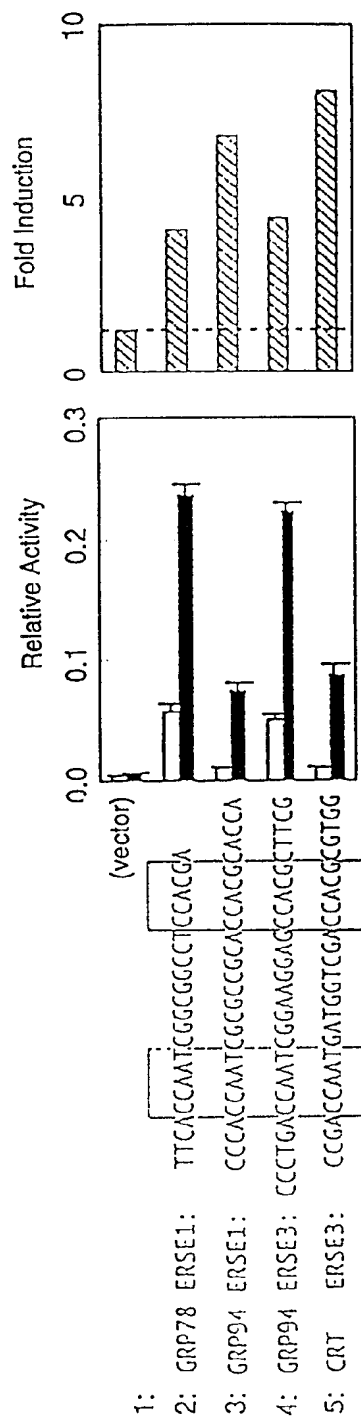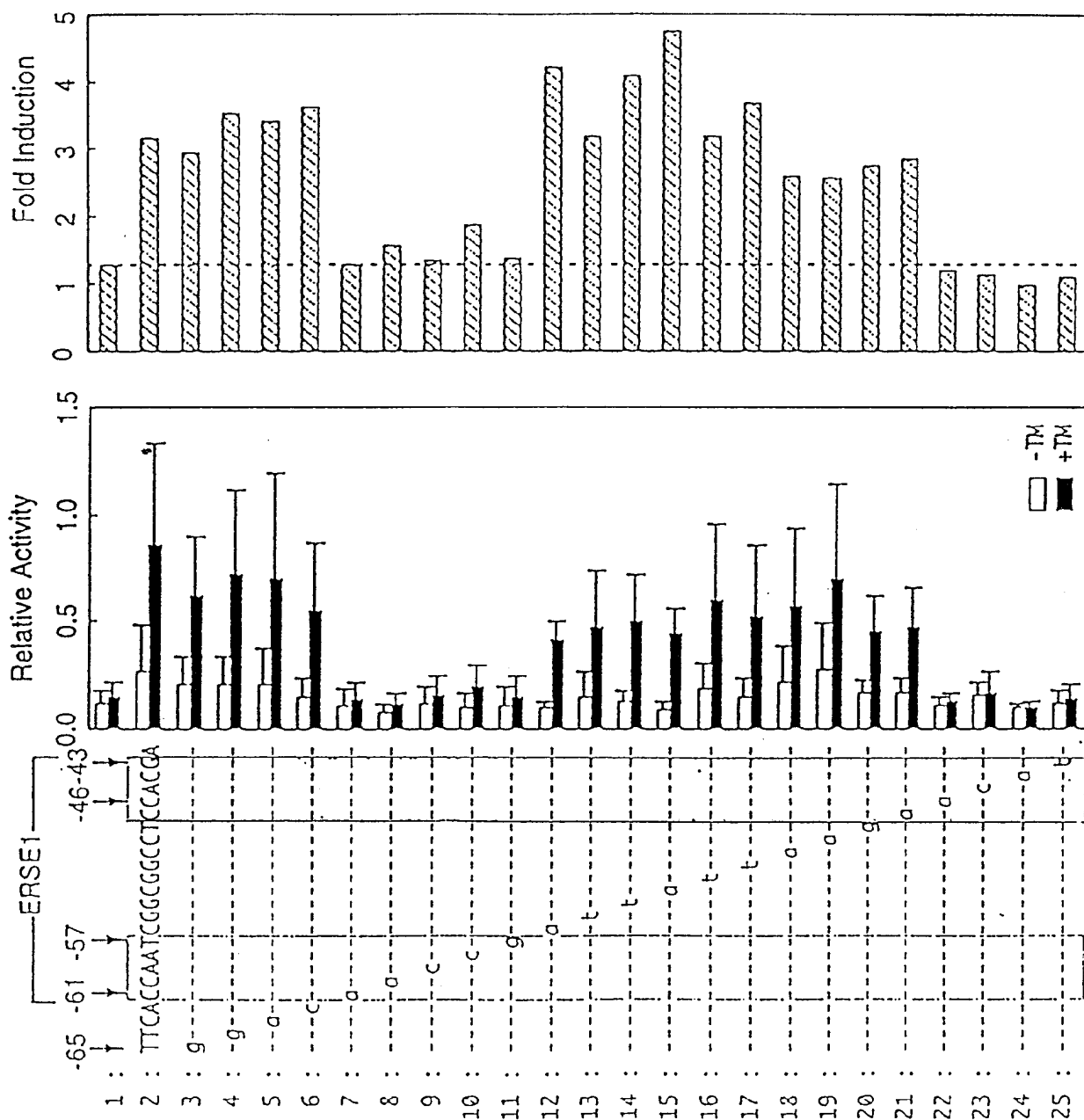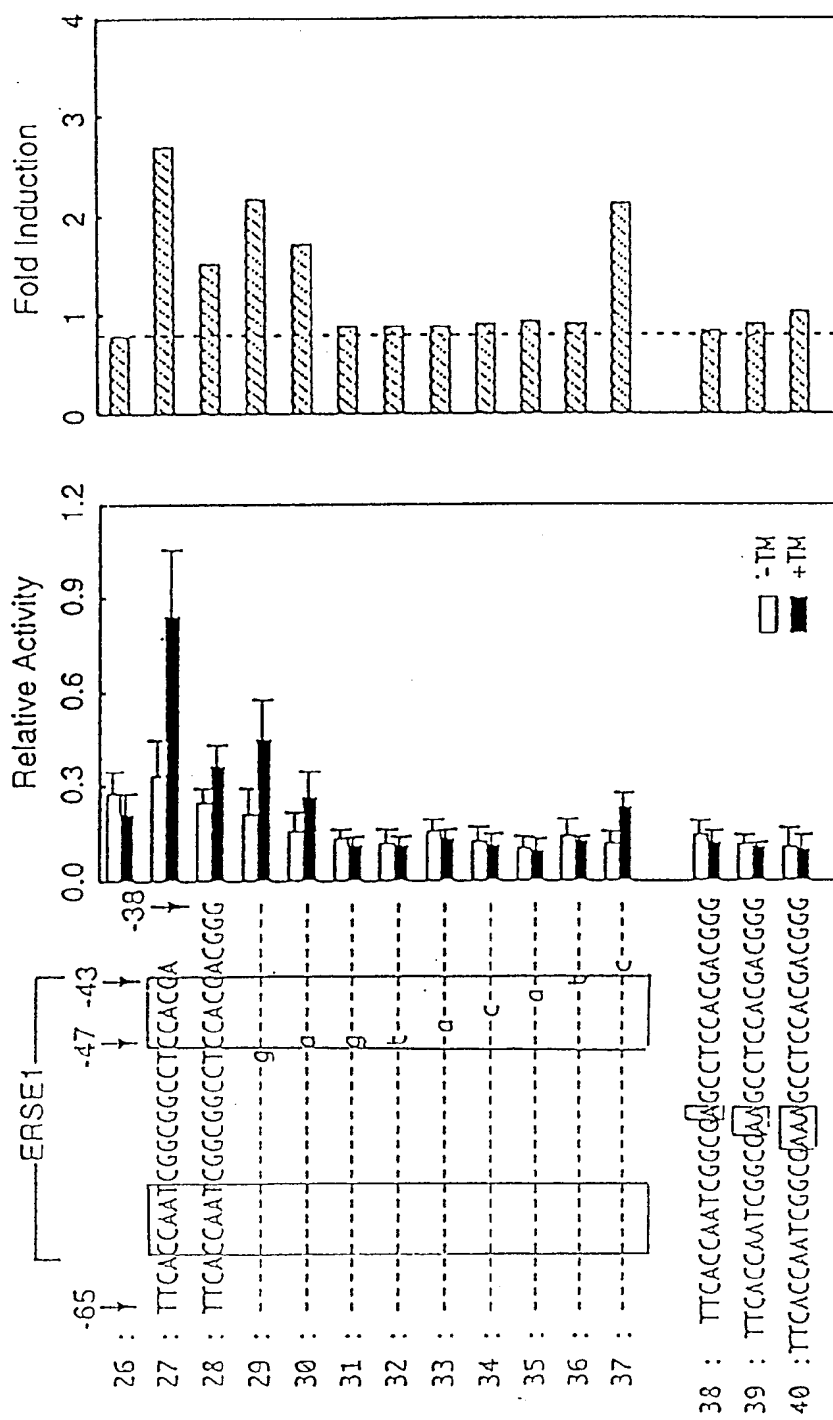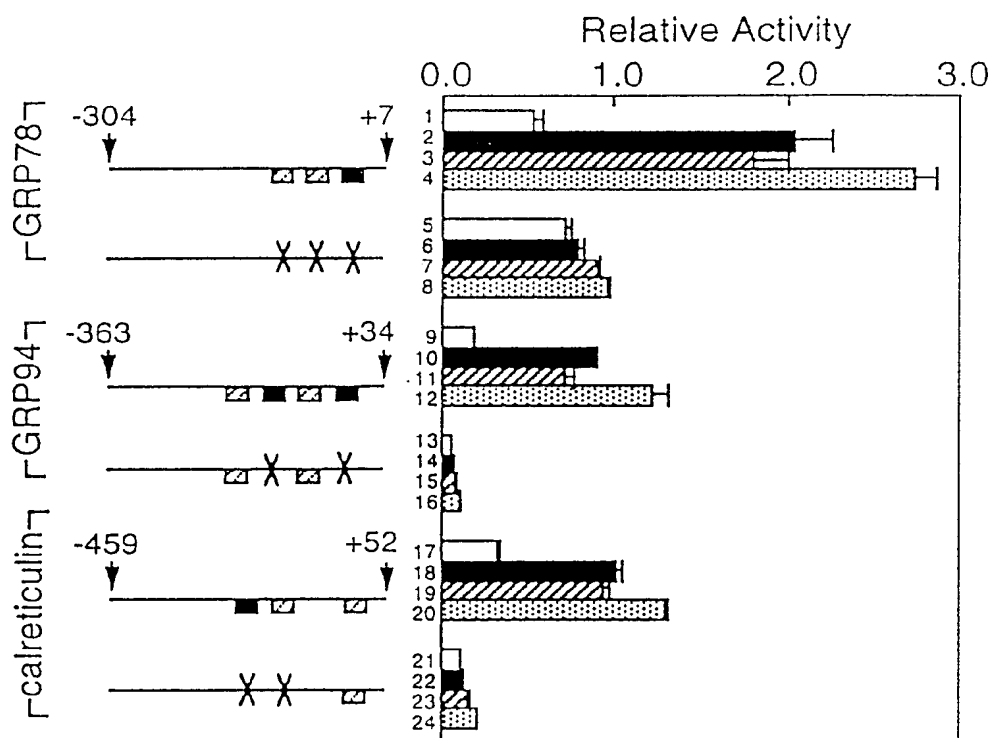
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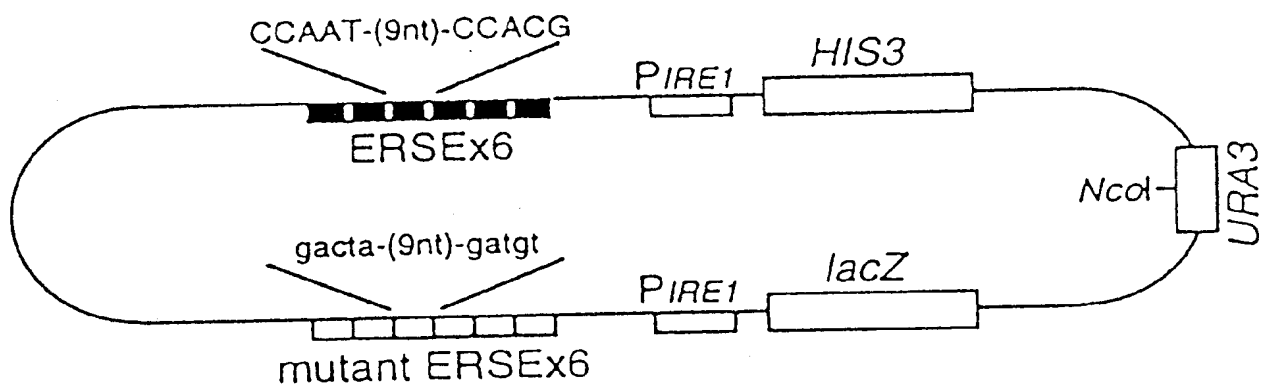
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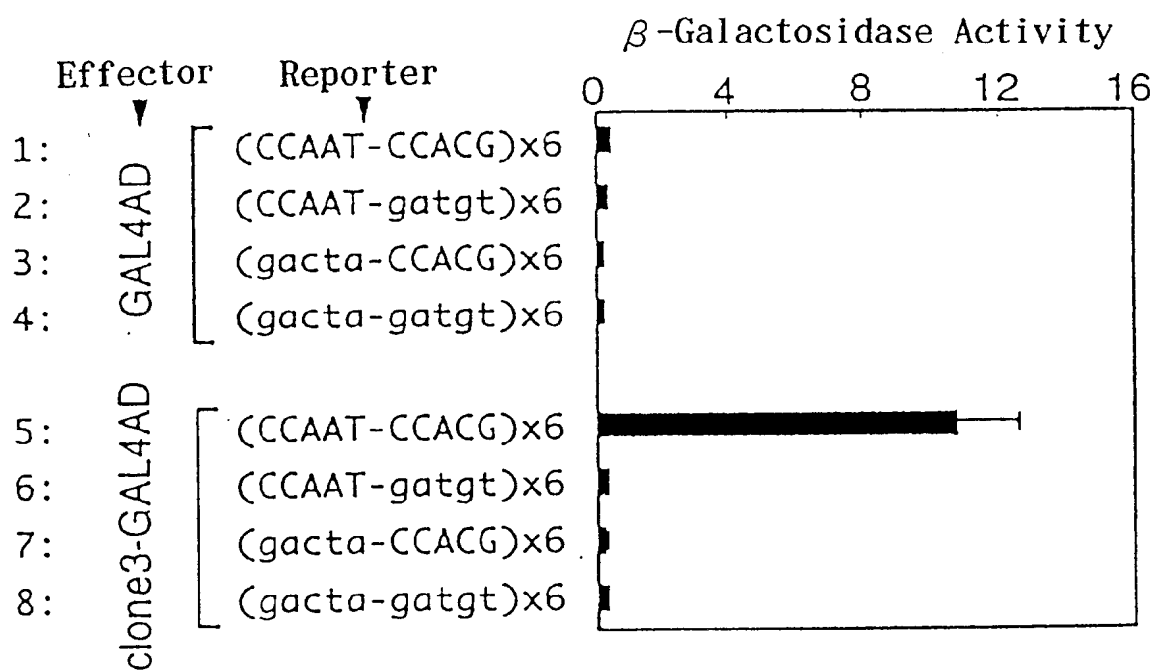
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CRE-BP1/ATF2　KRRKFLERNRAAASRCRQKRKVW
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c-fos(ATF5)　　EKENPKERNKMAAAKCRNRRREL

(C)

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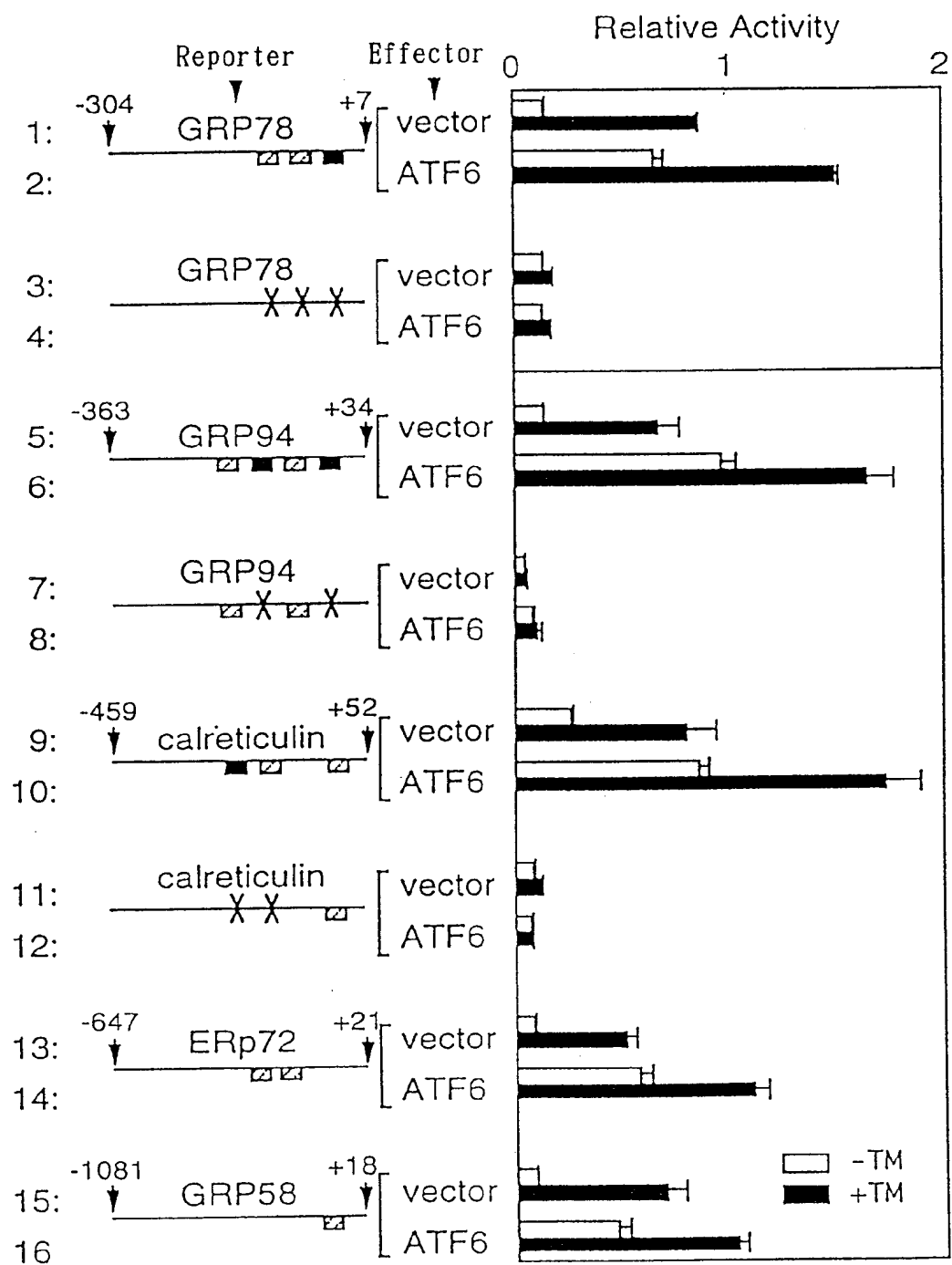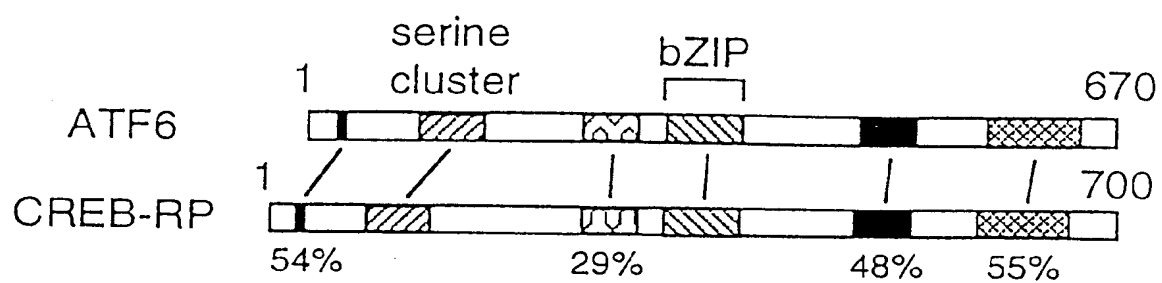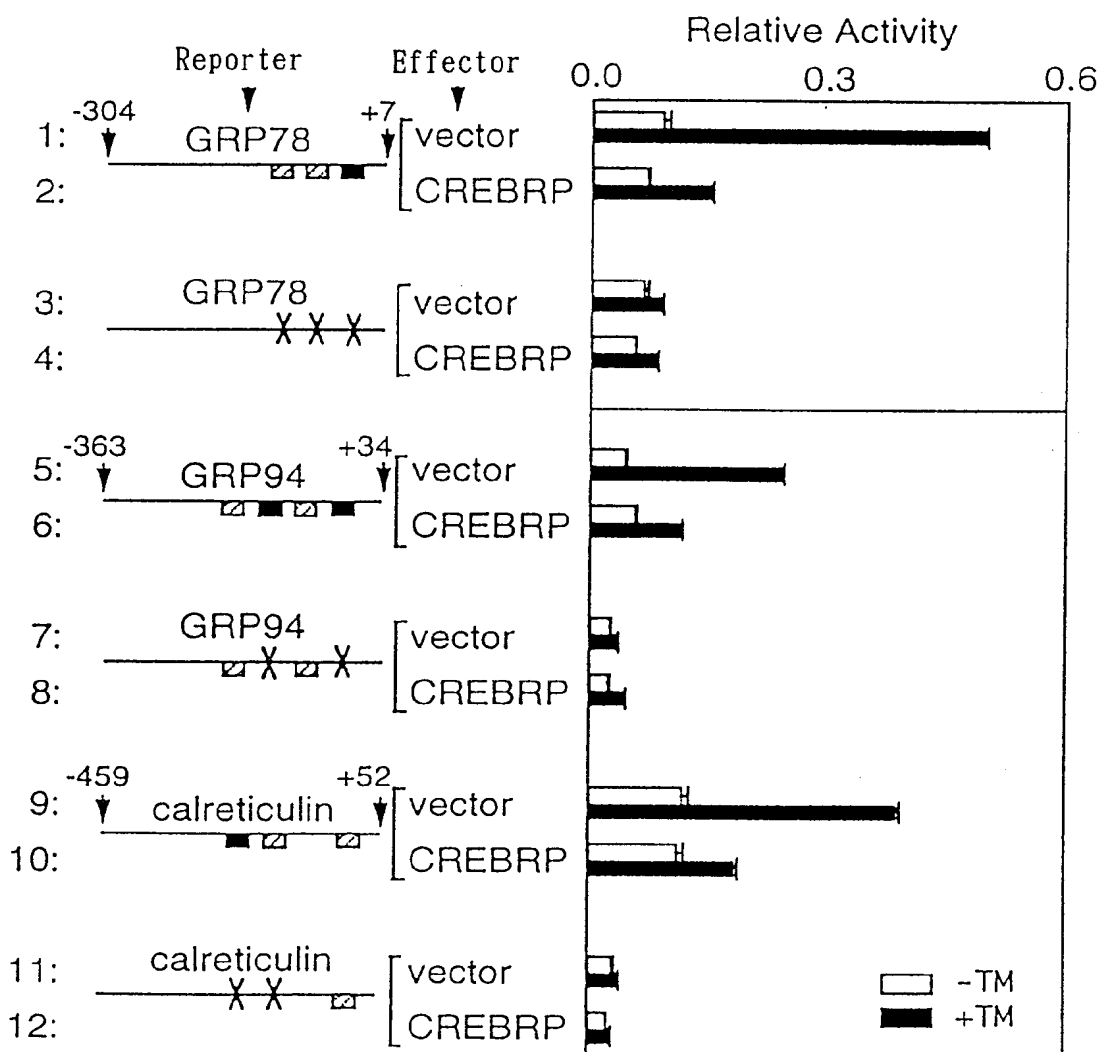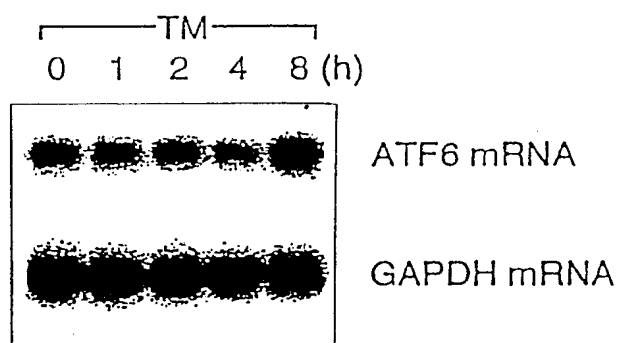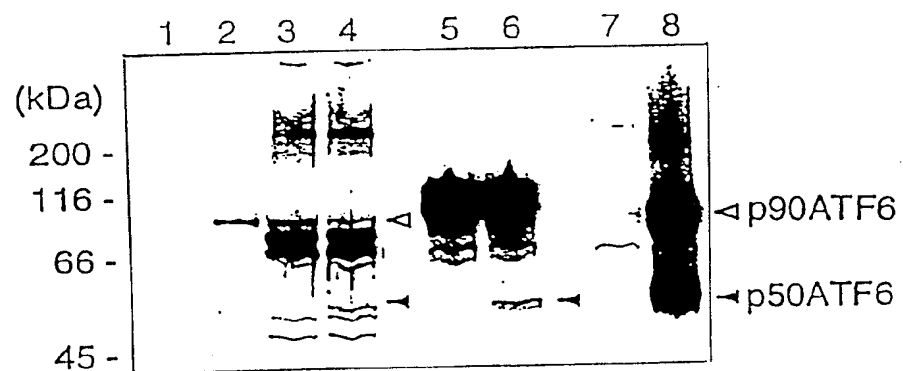
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F I G.   1 6

F I G． 1 7

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F I G . 2 0

F I G . 2 1

HeLa cells (W)

homogenized
1,000 x g 10 min

Nuclear Pellet (N)   Supernatant (S)

100,000 x g 1 hr

Cytosol (C)   Membranes (M)

F I G . 2 2

F I G . 2 3

F I G.   2 4

F I G . 2 5

F I G . 2 6

F I G . 2 7

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2	ATF6 (670)	
3	ATF6 (402)	
4	ATF6 (373)	
5	ATF6 (366)	
6	ATF6 (330)	
7	ATF6 (151-670)	
8	ATF6 (151-402)	
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10	ATF6 (151-366)	
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☐ TM(-)
■ TM(+)

Relative Activity
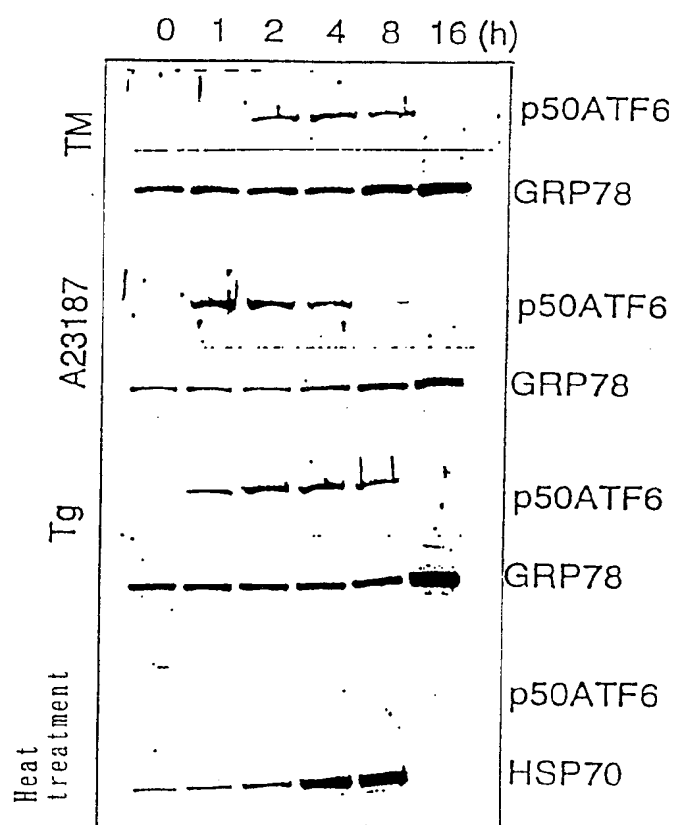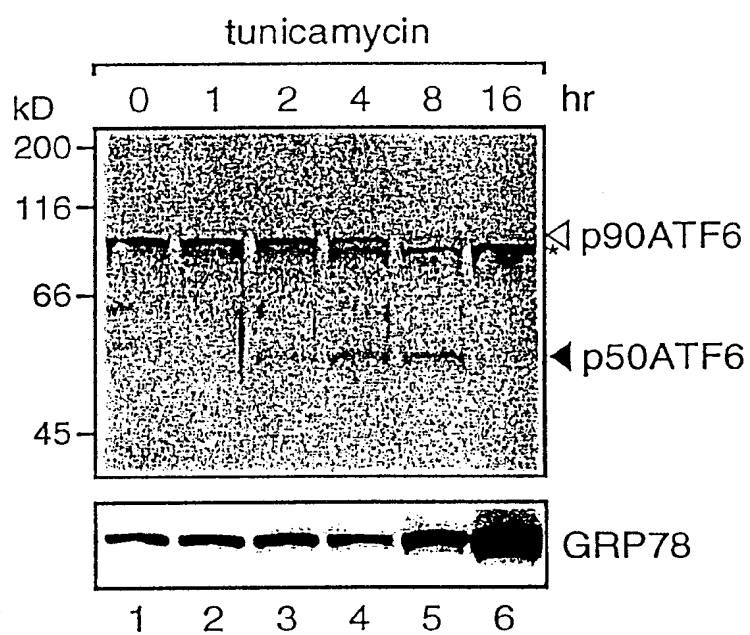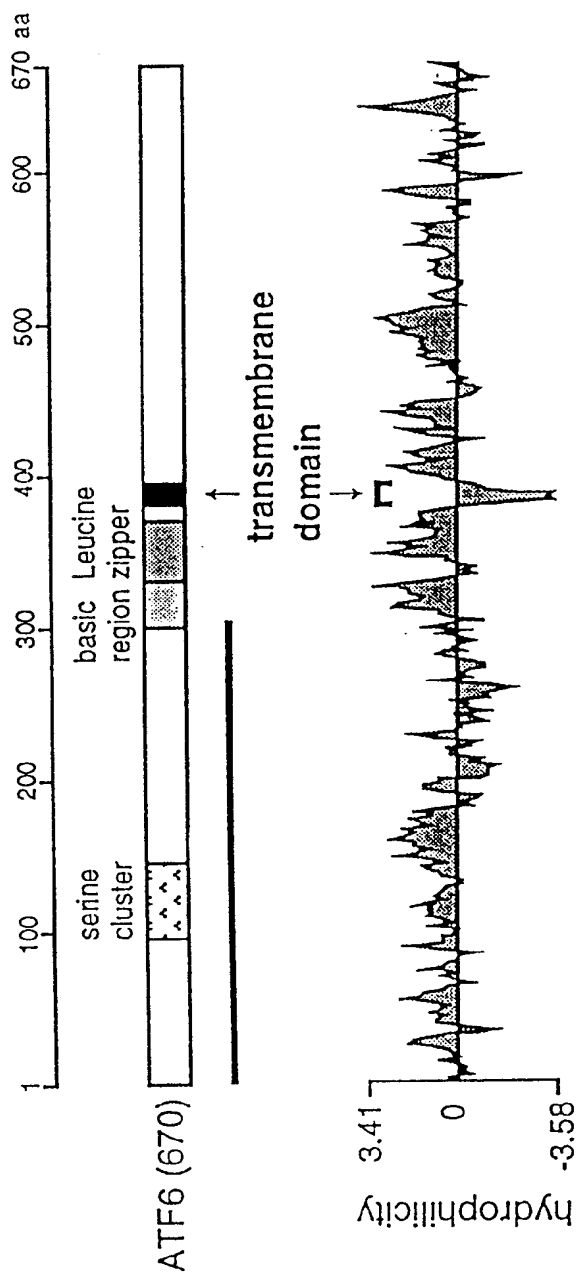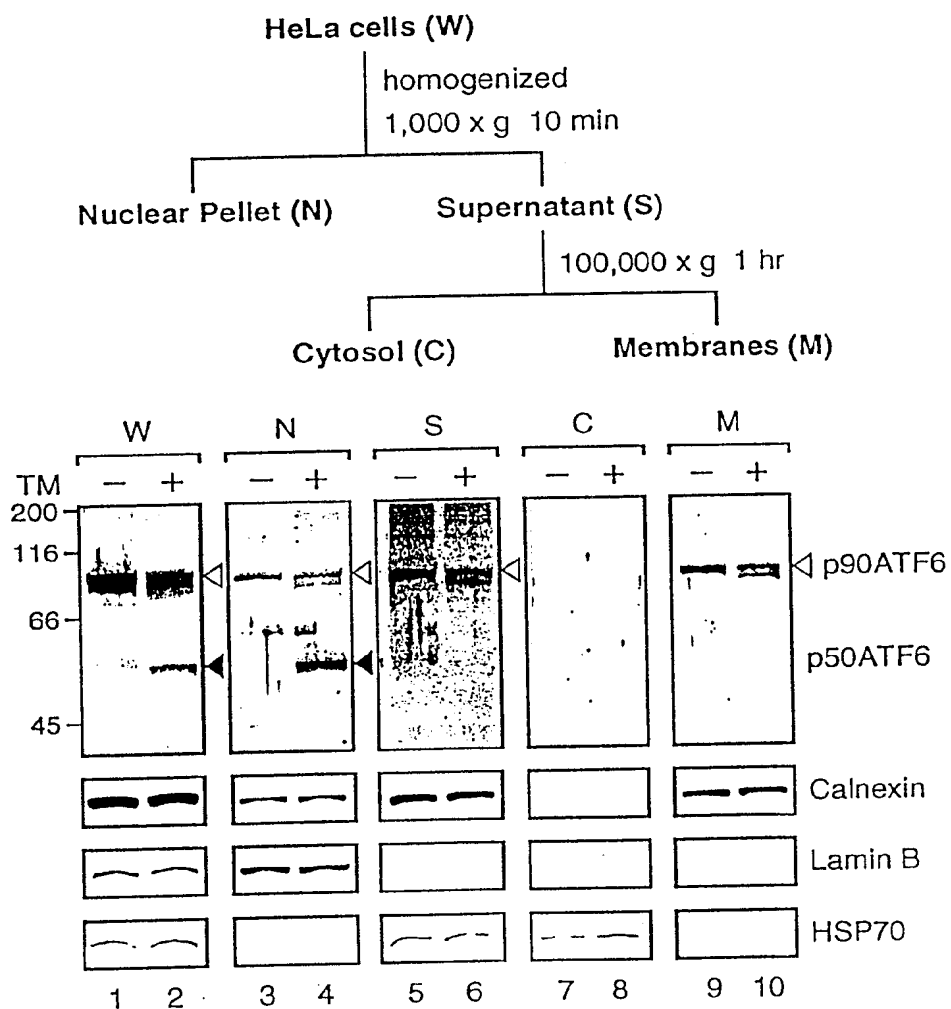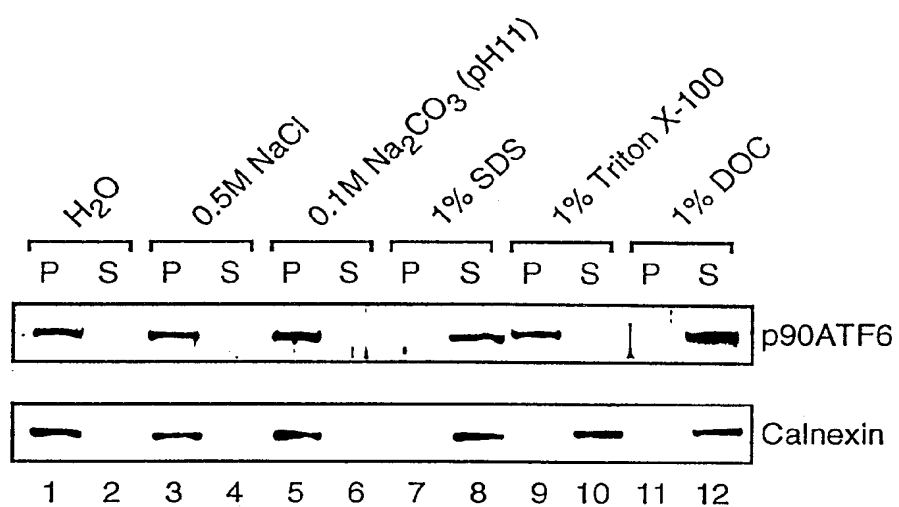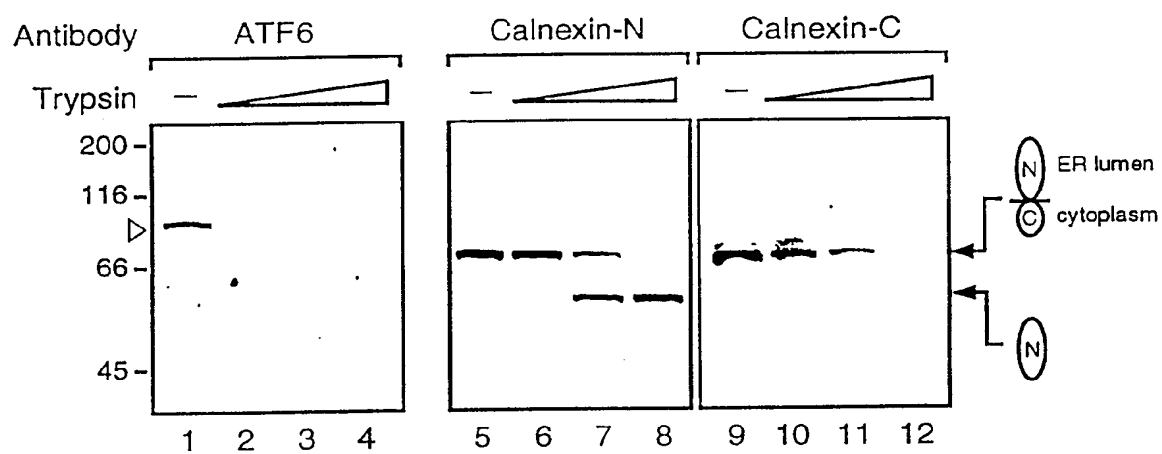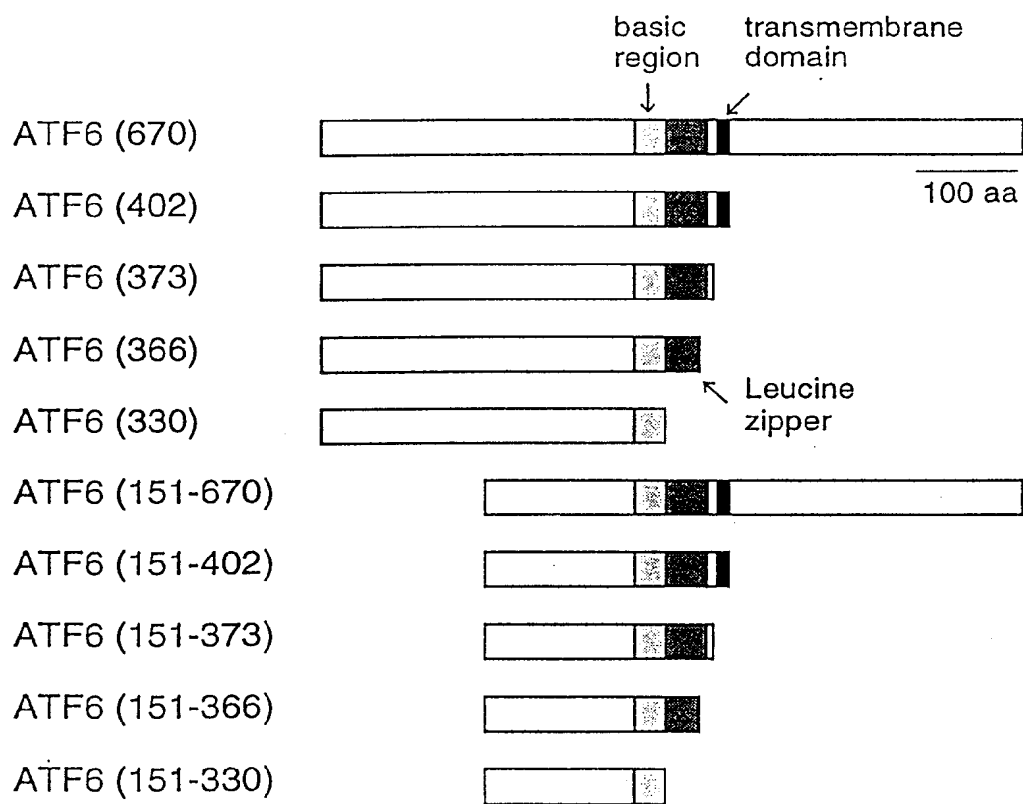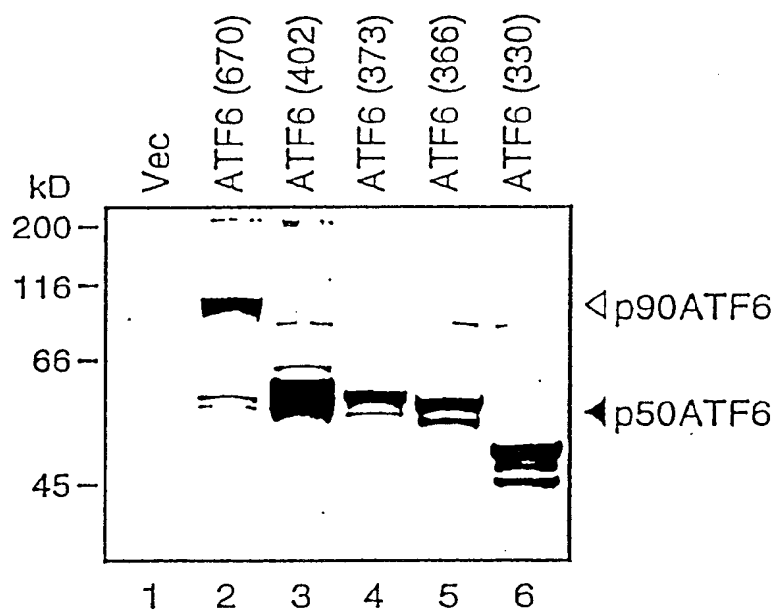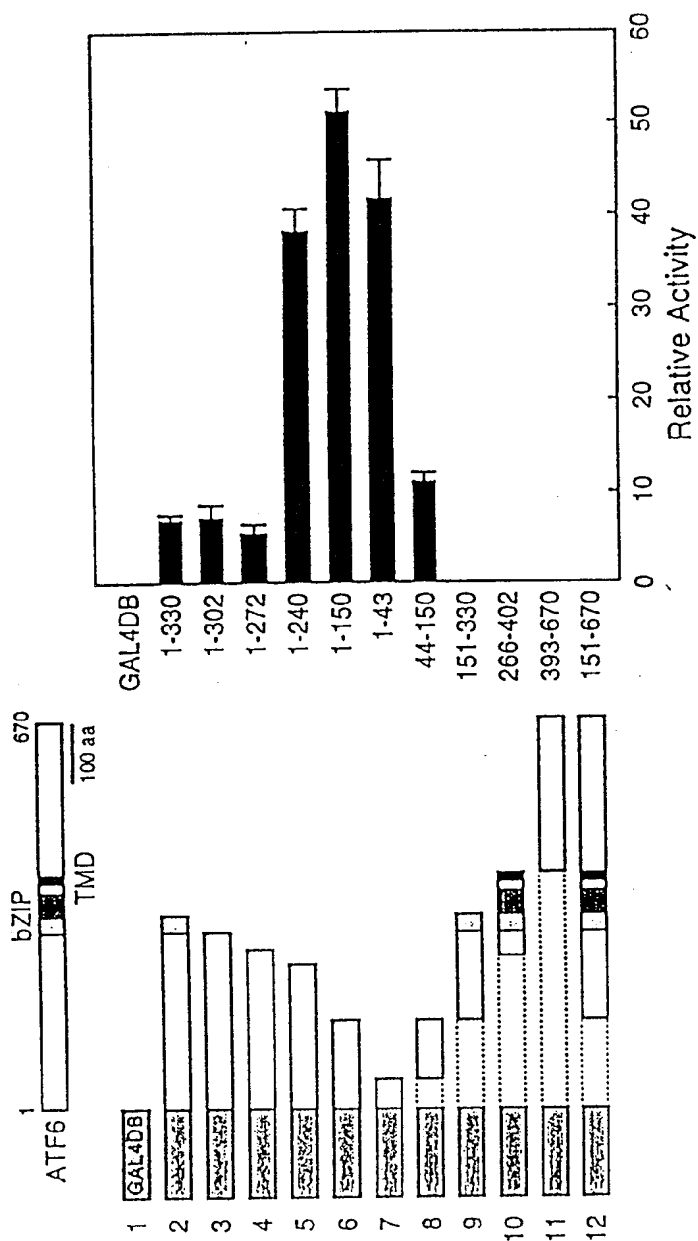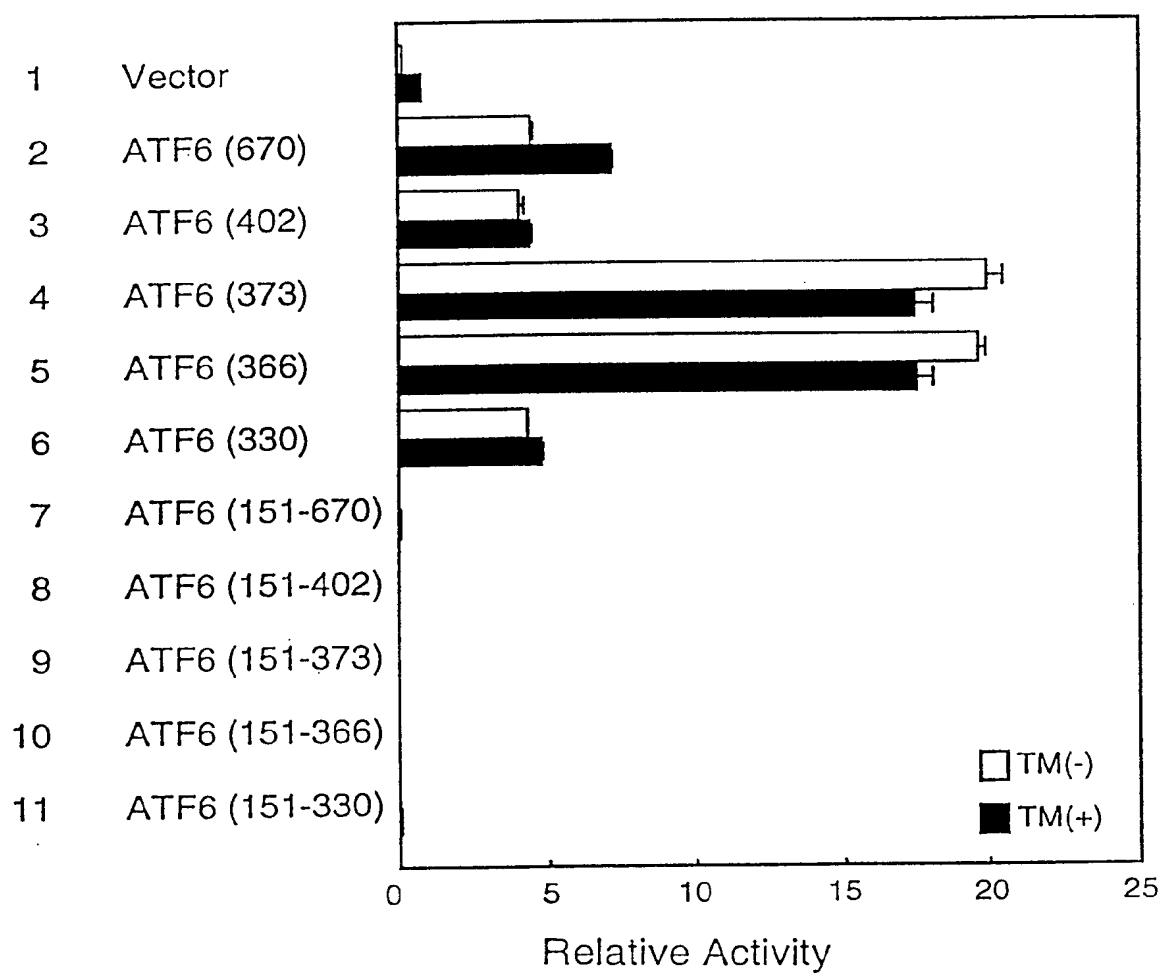
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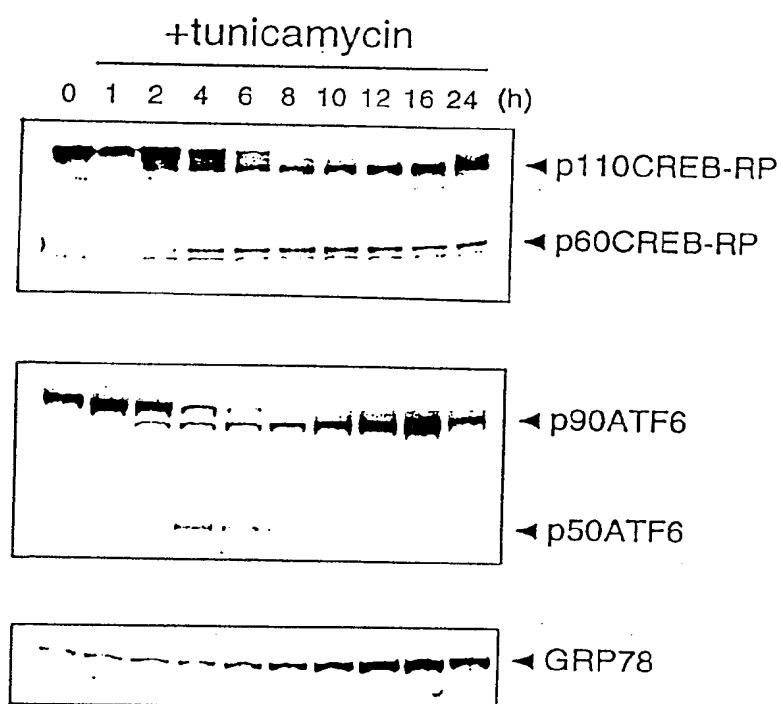
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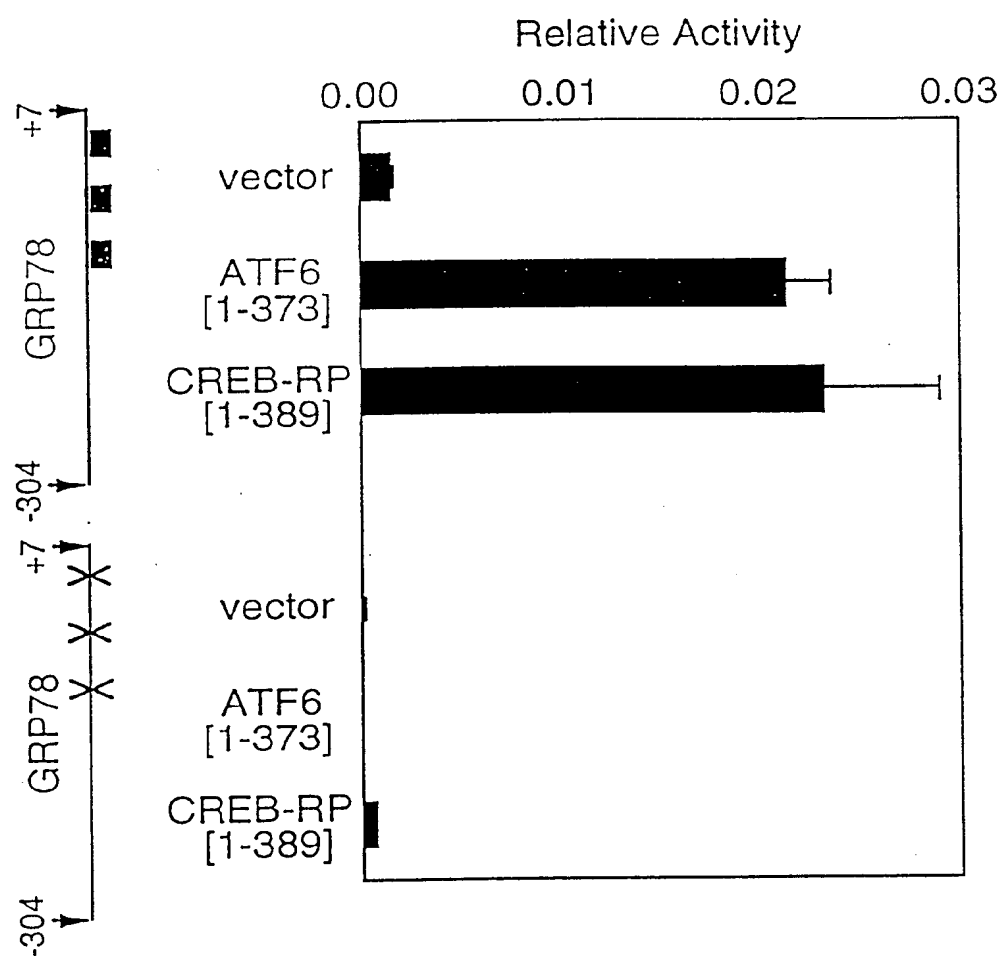
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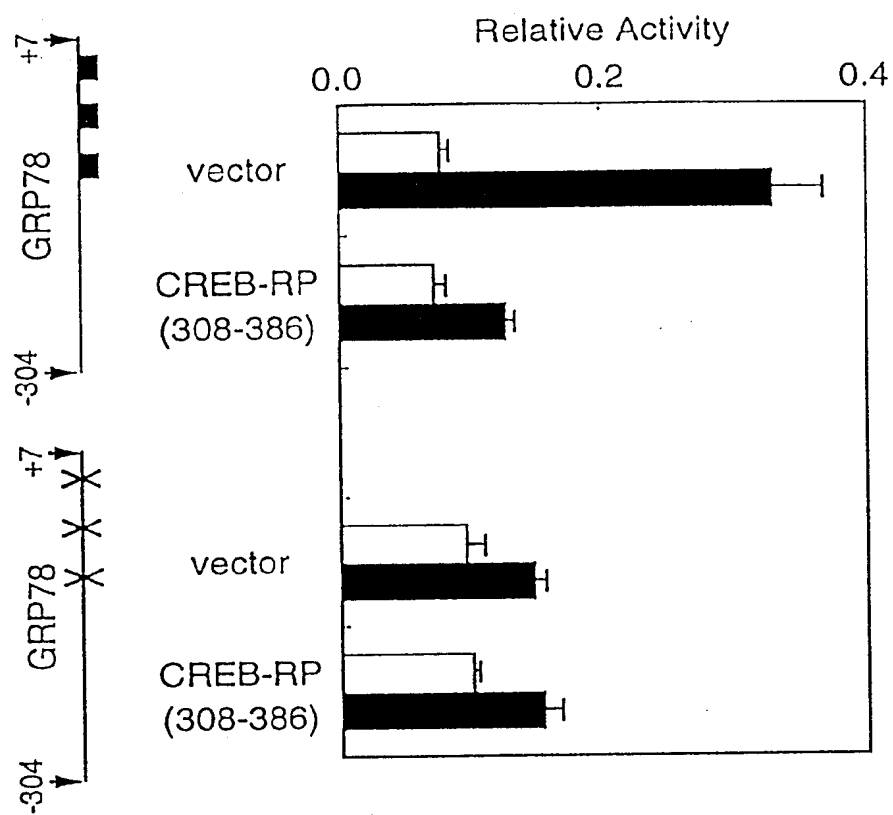
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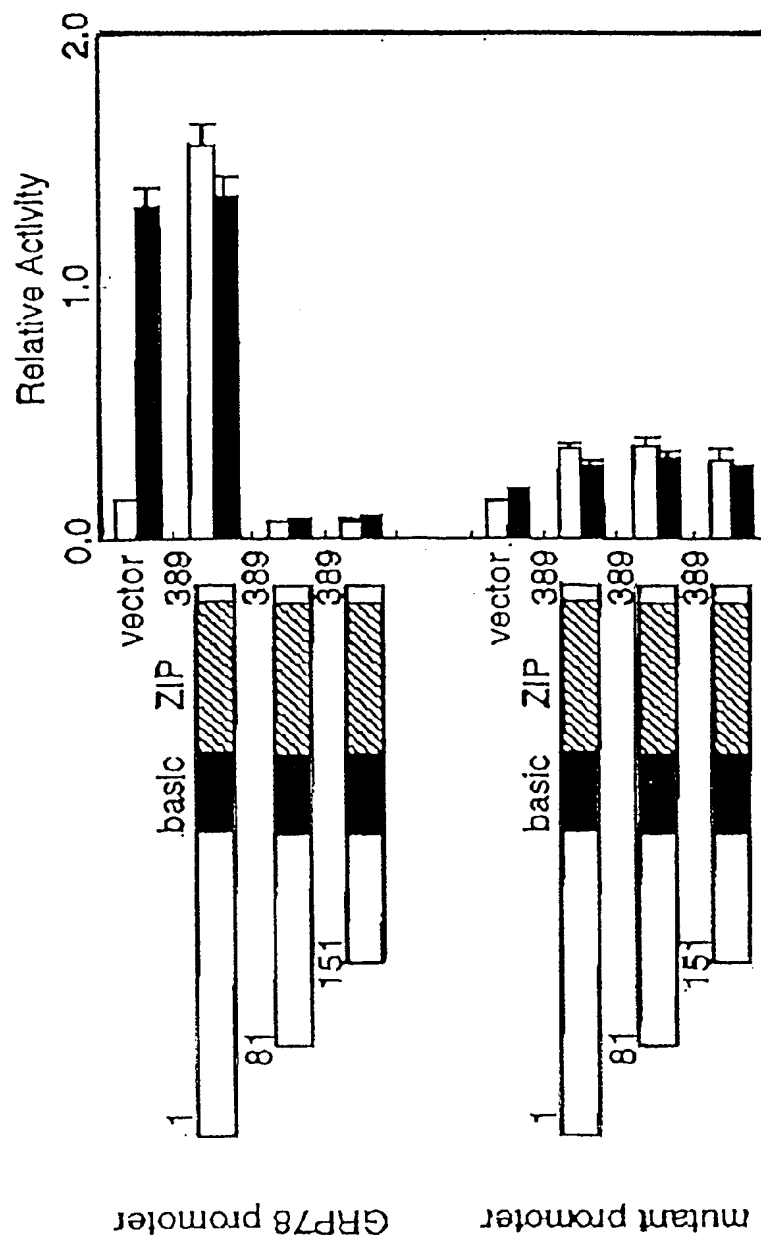
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Lys Ser Arg Arg Met Thr Asn Asn Gln Gln Lys Thr Arg Ile Leu
            500                 505                 510
Gln Gly Ala Leu Glu Gln Gly Ser Asn Ser Gln Leu Met Ala Val
            515                 520                 525
Gln Tyr Thr Glu Thr Thr Ser Ser Ile Ser Arg Asn Ser Gly Ser
            530                 535                 540
Glu Leu Gln Val Tyr Tyr Ala Ser Pro Arg Ser Tyr Gln Asp Phe
            545                 550                 555
Phe Glu Ala Ile Arg Arg Arg Gly Asp Thr Phe Tyr Val Val Ser
            560                 565                 570
Phe Arg Arg Asp His Leu Leu Leu Pro Ala Thr Thr His Asn Lys
            575                 580                 585
Thr Thr Arg Pro Lys Met Ser Ile Val Leu Pro Ala Ile Asn Ile
            590                 595                 600
Asn Glu Asn Val Ile Asn Gly Gln Asp Tyr Glu Val Met Met Gln
            605                 610                 615
Ile Asp Cys Gln Val Met Asp Thr Arg Ile Leu His Ile Lys Ser
            620                 625                 630
Ser Ser Val Pro Pro Tyr Leu Arg Asp Gln Gln Arg Asn Gln Thr
            635                 640                 645
Asn Thr Phe Phe Gly Ser Pro Pro Ala Ala Thr Glu Ala Thr His
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Val Val Ser Thr Ile Pro Glu Ser Leu Gln
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665   670

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<211> 2620

<212> DNA

<213> human

<400> 33

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ggactgggac agcaccttgt attctggcct agatgaagtg gccgaggagc agacgcagct   180

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gcaactatat cgccacccag accgttcgca gccagcattc ttggatgcaa ttgaccgacg   1800

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cttatttgag gtgggggtgt tcacctctct tctcatccct ttatcagaat atagggctcc   2340

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gttactggga gtaagggagg gtggggtggg ggggagttca ggtttatgtg tgtgcatttc   2580

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Leu Tyr Ser Gly Leu Asp Glu Val Ala Glu Glu Gln Thr Gln Leu
                  35   40   45
Phe Arg Cys Pro Glu Gln Asp Val Pro Phe Asp Gly Ser Ser Leu
                  50   55   60
Asp Val Gly Met Asp Val Ser Pro Ser Glu Pro Pro Trp Glu Leu
                  65   70   75
Leu Pro Ile Phe Pro Asp Leu Gln Val Lys Ser Glu Pro Ser Ser
                  80   85   90
Pro Cys Ser Ser Ser Ser Leu Ser Ser Glu Ser Ser Arg Leu Ser
                  95   100   105
Thr Glu Pro Ser Ser Glu Ala Leu Gly Val Gly Glu Val Leu His
                  110   115   120
Val Lys Thr Glu Ser Leu Ala Pro Pro Leu Cys Leu Leu Gly Asp
                  125   130   135
Asp Pro Thr Ser Ser Phe Glu Thr Val Gln Ile Asn Val Ile Pro
                  140   145   150
Thr Ser Asp Asp Ser Ser Asp Val Gln Thr Lys Ile Glu Pro Val
                  155   160   165
Ser Pro Cys Ser Ser Val Asn Ser Glu Ala Ser Leu Leu Ser Ala
                  170   175   180
Asp Ser Ser Ser Gln Ala Phe Ile Gly Glu Glu Val Leu Glu Val
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Lys Thr Glu Ser Leu Ser Pro Ser Gly Cys Leu Leu Trp Asp Val
                  200   205   210

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Pro Ala Pro Ser Leu Gly Ala Val Gln Ile Ser Met Gly Pro Ser
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Leu Asp Gly Ser Ser Gly Lys Ala Leu Pro Thr Arg Lys Pro Pro
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Leu Gln Pro Lys Pro Val Val Leu Thr Thr Val Pro Met Pro Ser
                245             250             255
Arg Ala Val Pro Pro Ser Thr Thr Val Leu Leu Gln Ser Leu Val
                260             265             270
Gln Pro Pro Pro Val Ser Pro Val Val Leu Ile Gln Gly Ala Ile
                275             280             285
Arg Val Gln Pro Glu Gly Pro Ala Pro Ser Leu Pro Arg Pro Glu
                290             295             300
Arg Lys Ser Ile Val Pro Ala Pro Met Pro Gly Asn Ser Cys Pro
                305             310             315
Pro Glu Val Asp Ala Lys Leu Leu Lys Arg Gln Gln Arg Met Ile
                320             325             330
Lys Asn Arg Glu Ser Ala Cys Gln Ser Arg Arg Lys Lys Lys Glu
                335             340             345
Tyr Leu Gln Gly Leu Glu Ala Arg Leu Gln Ala Val Leu Ala Asp
                350             355             360
Asn Gln Gln Leu Arg Arg Glu Asn Ala Ala Leu Arg Arg Arg Leu
                365             370             375
Glu Ala Leu Leu Ala Glu Asn Ser Glu Leu Lys Leu Gly Ser Gly
                380             385             390
Asn Arg Lys Val Val Cys Ile Met Val Phe Leu Leu Phe Ile Ala
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Phe Asn Phe Gly Pro Val Ser Ile Ser Glu Pro Pro Ser Ala Pro
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Ile Ser Pro Arg Met Asn Lys Gly Glu Pro Gln Pro Arg Arg His
            425                 430                 435
Leu Leu Gly Phe Ser Glu Gln Glu Pro Val Gln Gly Val Glu Pro
            440                 445                 450
Leu Gln Gly Ser Ser Gln Gly Pro Lys Glu Pro Gln Pro Ser Pro
            455                 460                 465
Thr Asp Gln Pro Ser Phe Ser Asn Leu Thr Ala Phe Pro Gly Gly
            470                 475                 480
Ala Lys Glu Leu Leu Leu Arg Asp Leu Asp Gln Leu Phe Leu Ser
            485                 490                 495
Ser Asp Cys Arg His Phe Asn Arg Thr Glu Ser Leu Arg Leu Ala
            500                 505                 510
Asp Glu Leu Ser Gly Trp Val Gln Arg His Gln Arg Gly Arg Arg
            515                 520                 525
Lys Ile Pro Gln Arg Ala Gln Glu Arg Gln Lys Ser Gln Pro Arg
            530                 535                 540
Lys Lys Ser Pro Pro Val Lys Ala Val Pro Ile Gln Pro Pro Gly
            545                 550                 555
Pro Pro Glu Arg Asp Ser Val Gly Gln Leu Gln Leu Tyr Arg His
            560                 565                 570
Pro Asp Arg Ser Gln Pro Ala Phe Leu Asp Ala Ile Asp Arg Arg
            575                 580                 585
Glu Asp Thr Phe Tyr Val Val Ser Phe Arg Arg Gly His Leu Leu
            590                 595                 600
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Leu Pro Ala Ile Ser His Asn Lys Thr Ser Arg Pro Lys Met Ser
             605   610   615

Leu Val Met Pro Ala Met Ala Pro Asn Glu Thr Leu Ser Gly Arg
             620   625   630

Gly Ala Pro Gly Asp Tyr Glu Glu Met Met Gln Ile Glu Cys Glu
             635   640   645

Val Met Asp Thr Arg Val Ile His Ile Lys Thr Ser Thr Val Pro
             650   655   660

Pro Ser Leu Arg Lys Gln Pro Ser Pro Thr Pro Gly Asn Ala Thr
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Gly Gly Pro Leu Pro Val Ser Ala Ala Ser Gln Ala His Gln Ala
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Ser His Gln Pro Leu Tyr Leu Asn His Pro
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<212> DNA
<213> Artificial Sequence

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<210> 36
<211> 29
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<213> human

<400> 36

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